HUMAN OSTEOARTHROSIS:

LECTIN AND HISTOCHEMICAL

STUDIES

OF KNEE

ARTICULAR CARTILAGE

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Declaration:

The studies making up this thesis were carried out at the Department of Pathological Sciences, Stopford Building, Medical School, Oxford Road University of Manchester, Manchester, United Kingdom and the Department of Pathology, University of Tasmania Medical School, Collins St, Hobart, Tasmania, Australia.

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This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the Thesis, and to the best of the candidate's knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

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ABSTRACT:

Osteoarthrosis is a common condition affecting the articular cartilage of diarthrodial joints. This study was undertaken in two parts to review the lectin and histochemical staining characteristics of normal, aged and osteoarthrotic cartilage. Firstly, using a panel of histochemical stains the normal microanatomy of cartilage was reviewed with reference to the chondro-osseous junction. Secondly, using a panel of 19 lectins, carbohydrate expression of a number of parameters of the chondro-osseous sections was assessed. Three key findings were made in this study:

1. Ageing and early osteoarthrotic cartilage showed characteristic differences in their macroscopic and microscopic features. In particular ageing cartilage showed less loss of the normal viscoelastic response on indentation tests. At the microscopic level there was marked disruption of the normal chondrocyte architecture and chondro-osseous region and this was most pronounced in early osteoarthrosis.
2. The anatomy of the chondro-osseous region is more complex than previously documented. Clearly demonstrated were pegs of uncalcified cartilage faithfully followed by the tidemark dipping through calcified cartilage and abutting onto bone marrow spaces.
3. The main findings in relation to lectins were that: matrix staining was varied in normals in anatomical zones and regions and there were characteristic alterations in ageing and osteoarthrosis; chondrocyte cytoplasm and membrane staining was not always present suggesting that some cells may be metabolically active and others in a non-responsive phase; zone V matrix and uncalcified cartilage pegs exhibited a different staining pattern to the normal matrix indicating possible differences in function.

Three main conclusions were made from these findings. Firstly, that the pathophysiological processes in ageing and early osteoarthrosis are fundamentally different, one being a natural physiological phenomena and the other exhibiting the hallmarks of a progressive disease process. Secondly, that the chondro-osseous junction region and tidemark remain poorly understood anatomical regions. The presence of interdigitating uncalcified cartilage pegs which connect with underlying bone marrow spaces suggesting this region may have a significant role in nutrition and possibly in the evolution of osteoarthrosis. Thirdly, the carbohydrates of cartilage glycoproteins play a

significant role in the microenvironment of articular cartilage and subchondral bone and our current knowledge of the carbohydrate chemistry of cartilage is insufficient.

That articular cartilage provides a unique role in maintaining mobility is demonstrated by the incapacity and morbidity that results from osteoarthrosis. A greater understanding of the chondro-osseous region and of cartilage carbohydrate chemistry may enhance our understanding of osteoarthrosis and assist in developing treatment strategies for this common articular disease.

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LIST OF ABBREVIATIONS

AB	Alcian blue
AM	Acellular matrix
CC	Chondroclasts
CH	Chondrocyte
CJ	Chondro-osseous junction
CL	Chondrolysis
CM	Cell membrane
CY	Cytoplasm
EO	Endochondral ossification
FI	Fibrosis
FN	Fat necrosis
FT	Frequency of chondrocyte clones
Fuc	Fucose
Gal	Galactose
Gal NAc	N-acetyl galactosamine
GalNAc/GalNAc	N-acetylchitobiose
GalNAc/GlcNAc	N-acetyl lactosamine
Glc	Glucose
GlcNAc	N-acetyl glucosamine
GR	Overall grade scored
GSA 1	Lectin from Griffonia simplicifolia
H&E	Haematoxylin and eosin
HL	Horizontal cartilage splits
HS	Horizontal split tide mark
HY	Hypertrophic chondrocytes
IC	Intraclonal matrix
IM	Interterritorial matrix
MA	Matrix
Man	Mannose
MF	Matrix fibrosis
MH	Horizontal microfracture
MNGC	Subchondral multinucleate giant cells
MO	Matrix overall staining
MV	Vertical microfracture
NB	New bone
NC	New cartilage
NeuNAc	N-acetyl neuraminic acid (sialic acid)
OA	Osteoarthrosis
OB	Osteoblasts
OC	Osteoclasts
OS	Osteocytes
PAFIB	Overlying fibrosis

PC	Pericellular
PG	Chondrocyte pegs
PSR	Picrosirius red
RCA 1	Ricinis communis (casterberry)
S	Surface staining
SCBP	Subchondral bone plate
SF	Surface fibrosis
SIA	Sialic acid
SO	Safranin O
SP	Splits
SU	Surface fibrillation
TB	Toluidine blue
TIMP	Tissue inhibitor of metalloproteinase
TD	Tide mark re-duplication
TM	Territorial matrix
VE	Vessels
VI	Vascular invasion at tide mark
VL	Vertical cartilage splits
VM	Matrix around vessel
WB	Woven bone
Z1	Zone 1 cartilage
ZII	Zone 2 cartilage
ZIII	Zone 3 cartilage
ZIV	Zone 4 cartilage
ZV	Zone 5 cartilage
ZP	Zona splendens

LECTINS USED IN THIS STUDY

Acronym	Source	
AHA	Arachis hypogaea	Peanut
BSA	Griffonia simplicifolia isolectin (B4/II)	-
CON A	Canavalia ensiformis	Jack bean
CTA	Erythrina corallendron	Coral tree
DBA	Dolichos biflorus	Horse ground
DSA	Datura stramonium	Jimson weed
ECA	Erythrina cristagalli	Coral tree
GNA	Galanthus nivalis	Snow drop
HAA	Helix aspersa	Garden snail
HPA	Helix pomatia	Roman /Edible snail
L/E-PHA	Phaseolus vulgaris (leucoagglutinin)	Kidney bean
LCA	Lens culinaris	Common lentil
LEA	Lycopersicon esculentum	Tomato
LFA	Limax flavus	Yellow slug
LTA	Tetragonolobus pureus	Lotus
MAA	Maakia amurensis	-
MPA	Maclura pomifera	Osage orange
NPA	Narcissus pseudonarcissus	Daffodil
PSA	Pisum sativum	Garden pea
PWM	Phytolacca americana	Pokeweed mitogen
SBA	Glycine max	Soy bean
SNA	Sambucus nigra	Elderberry bark
STA	Solanum tuberosum	Potato
UEA-1	Ulex europaeus –1	Gorse
VVAB4	Vivia villosa	Hairy vetch
WFA	Wisteria floribunda	Wisteria
WGA	Wheat germ agglutinin	

INTRODUCTION:

Osteoarthrosis is the most common form of arthritis and is a major cause of morbidity and disability in the community, imposing a substantial financial burden on public and private health care services. The understanding of the pathogenesis of osteoarthrosis has improved over the past 25 years, however, the precise molecular causes remain to be elucidated. It is undoubtedly a complex pathophysiological process involving genetic, environmental, metabolic and biochemical factors. In order to understand the pathology of osteoarthrosis it is vital to understand the normal structure of articular cartilage and the natural physiological changes that occur in ageing. Articular cartilage is a unique biological material composed of a three dimensional mesh of type II and lesser cross-linking collagens, the resulting spaces containing highly hydrated, aggregated proteoglycan macromolecules. This unique ultrastructure imparts the characteristic biomechanical property of viscoelasticity on the cartilage.

Normal articular cartilage undergoes a gradual degradative process as part of the normal physiological process of ageing. The pathophysiology of ageing and early osteoarthrosis have often been likened, however, the differences have not been clearly characterised. The normal anatomy of the cartilage matrix and subchondral bone is well described. That of the chondro-osseous region delineated by the tidemark (uncalcified-calcified cartilage boundary) and the cement line (calcified cartilage – osteochondral bone boundary) has not been well defined. This region has always been considered to be a relatively inert area and its role in cartilage metabolism and nutrition has escaped close study. It has always been considered that cartilage nutrition comes predominantly through transfer of substances at the cartilage surface. Simple lower power light microscopic examination of normal cartilage demonstrate pegs of uncalcified cartilage which dip into the calcified zone adjacent to underlying subchondral bone marrow spaces. A detailed study using classical histological stains was undertaken to characterise the morphological features of ageing and early osteoarthrotic cartilage and the normal anatomy of the tidemark. Three dimensional reconstructive studies of the chondro-osseous region were performed to more clearly define the microanatomy. An additional facet of the research were S-100 protein studies as this molecule is involved in calcification it might provide information about the tidemark region.

The carbohydrate residues of cartilage and subchondral bone have not been well defined in either normal or osteoarthrotic joints. The glycoproteins of the matrix and chondrocyte cytoplasm and cell membrane are important structural molecules involved in the orientation of macromolecules including enzymes and cell receptors. Subtle perturbations in the carbohydrate biochemistry can be detected with lectins which are a group of sugar binding proteins that stain a wide selection of saccharide residues. Therefore changes in staining patterns in normal, ageing and osteoarthrotic cartilage provides information in relation to structural alterations in glycoproteins. Extensive histochemical studies were undertaken using a broad panel of lectins taken from seven lectin groups which have an affinity for sugars in the cartilage matrix.

The principal aims of this study were:

- To undertake a comprehensive review of the structure of normal, ageing and osteoarthrotic human adult articular cartilage using a panel of classical histochemical stains, with particular reference to the chondro-osseous junction and to define morphological differences between ageing and osteoarthrotic cartilage.
- To study the normal tinctorial properties of the tidemark and to undertake 3D reconstruction of this region.
- To assess S-100 protein staining of cartilage.
- Using a battery of lectins categorise the expression of carbohydrate residues in normal, ageing and osteoarthrotic human articular cartilage.
- Interpret these findings and postulate their significance in osteoarthrosis.

1 CHAPTER ONE: ARTICULAR CARTILAGE; STRUCTURE, FUNCTION, AGEING AND OSTEOARTHROSIS

1.1 Introduction to Cartilage

The articular surfaces of synovial joints such as the knee are covered by hyaline cartilage and the low frictional resistance between opposing surfaces is enhanced by synovial fluid present within the joint cavity. Normal young cartilage is smooth, white and glistening but with advancing age it becomes yellowed, duller and somewhat thinner (Kraus 1997). Cartilage thickness in the normal adult may range from two to four millimetres and may vary across different regions within a joint depending upon the loading characteristics and dynamic interactions of that articulation. Functionally, articular cartilage acts to distribute and dampen loads over articulating regions and provides a low friction surface over which bones can move. Structurally, the hyaline cartilage matrix consists of a three dimensional collagen mesh in between which lie highly hydrated proteoglycans. Within this matrix are chondrocytes, which in the adult appear autonomous with no cell-cell connections. A distinctive line, the tidemark, separates hyaline cartilage the calcified cartilage, which then merges, into the underlying osteochondral bone. Both ageing changes and the cartilage degeneration in osteoarthrosis has been an area of growing research interest for a number of years. Interpretation of the staining patterns seen with classical and lectin histochemical techniques allow a greater understanding of these processes. This chapter reviews the current understanding of the anatomy of cartilage and its appearance in ageing and osteoarthrosis.

1.2 Matrix Structure and Function

Articular cartilage has excellent friction, lubrication and wear characteristics and serves as the load-bearing material of joints (Dowson 1981, Mow 1986). These essential mechanical characteristics allow articular cartilage to function with little damage or degenerative change over time providing normal physiological conditions remain unaltered. Damage due to trauma, impact injuries, abnormal joint loading or excessive wear, however, can lead to change in the composition, structure, and material properties of the tissue. Such physiological stressors can alter the ability of cartilage to survive

and function in the strenuous mechanical environment normally found in weight-bearing joints. (Radin 1976, Mankin 1984,Gardner 1991). Articular cartilage is one of the most widely studied of all connective tissues because of its pivotal role in the function and health of normal diarthroidal joints. Biochemical studies (Sokoloff 1979, Maroudas 1980, Muir 1980 & 1983, Miller 1985) show that the cartilage matrix consists of hydrophilic, negatively charged proteoglycan molecules entrapped in a three dimensional "chicken mesh" of fine collagen fibrils (Figure 1.1) This matrix consists of a supportive framework of type II collagen, with a small number of other structural collagens, VII and IX-XII. Entrapped between this complex collagen network are highly hydrated aggregating type proteoglycans. The resulting cytoskeleton of the cartilage is complex, highly organised and non-homogenous. The ultrastructure and composition of the cartilage varies within the joint in depth, location and age. Specialised cells, the chondrocytes, which are distributed throughout the matrix, produce this matrix.

Histological and morphological studies have shown the anisotropic and non-homogeneous nature of articular cartilage (Bullough 1968, Clarke 1971, Redler 1974, Lane 1975, 1975, Eyre 1980, Broom 1983, Ratcliffe 1984, Poole 1984, Hunziker 1987). Studies of composition, structure and material properties have revealed the mechanism by which components of the matrix may interact to provide articular cartilage with its mechanical properties in tension, compression and shear (Kempson 1971, 1973 and 1979, Woo 1976 and 1987, Mow 1980, 1982 and 1984A, Armstrong 1982). In addition, this composition and structure of articular cartilage also provides the physiochemical mechanisms that regulate tissues swelling and fluid and ion-transport properties (Lai 1989, 1991, Akizuki 1987, Maroudas 1971, 1973, 1976, Myers 1984). These properties lead to the intrinsic mechanical properties of the collagen-proteoglycan solid matrix and the deformational behaviour of cartilage. An increased understanding of the biochemical and physical properties of cartilage have led to the development of the bi-phasic theory. This theory describes the material properties and mechanical behaviour of cartilage as being viscoelastic (Mow 1980, 1982, 1984B) and behaving as two immiscible liquids, one solid and the other fluid. Through this fundamental understanding of articular cartilage the relationships between the composition, structure and mechanical behaviour of normal cartilage can be determined. The corresponding relationships are now being developed in ageing and osteoarthrotic cartilage (Muir 1977).

The solid phase consists of collagens, non-collagenous proteins, glycoproteins and proteoglycans representing approximately 20% of the tissue by wet weight. The fluid phases chiefly water and dissolved inorganic salts, saturates this solid matrix and represents the remaining 80% of the tissue. These separate components are now reviewed in normal cartilage before considering the pathology of ageing and osteoarthrosis.

1.2.1 Collagens

Cartilage, like most connective tissues contains several genetically distinct types of collagen (Burgeson 1988, Grant 1988, Mayne 1989, Gannon 1991, Kielty 1992). The primary collagen of articular cartilage type II, accounts for 90%-95% of the total tissue collagen content and forms the cross-banded fibrillar structures noted by electromicroscopy (Bucklewater 1988, Nimni 1988). Type II collagen is secreted by the chondrocytes as procollagen molecules, which consist of 3 alpha I polypeptide chains organised into a triple helix. These molecules after organisation unite to form fibrils, which subsequently develop covalent cross-links to form the structural framework of the articular cartilage (Eyre 1988,1991,Ricard-Blund 1989). The minor collagens of articular cartilage are believed to make important contributions to the structure of the matrix. For example, type IX collagen, a short non-fibrillar collagen, binds covalently to type II collagen fibrils and may help link fibrils together or bind them to other matrix molecules (Bruckner 1985, Eyre 1987, Van der Rest 1988). Type XI collagen, a minor fibrillar collagen may be involved in controlling the diameter of the type II fibrils (Ard 1986). Other collagens, including type V and VI may form part of the matrix, but their function and location in the matrix molecular framework is as yet poorly understood. The most important mechanical properties of collagens fibres are their tensile stiffness and strength, readily apparent in tissues such as ligaments and tendons which have high proportions of parallel collagen fibres. In cartilage these mechanical properties are provided by the normal three-dimensional inter-relationship between the different collagens. In ageing and osteoarthrosis there is a break down of cross-links in this 3D mesh leading to an alteration in the cartilage stereochemistry with increased hydration of proteoglycans and an alteration in the visco-elastic mechanical properties of the cartilage. These changes at the light microscope level are reflected in altered staining properties (Lippiello 1977, Adam 1983, Eyre 1991).

1.2.2 Proteoglycans

These are complex glycoproteins, consisting of a protein core to which glycosaminoglycans protein chains are attached. They constitute the second largest portion of the solid phase in articular cartilage accounting for 5% - 10% of the wet weight (Maroudas 1980, Muir 1983). Proteoglycans of hyaline cartilage matrix are mainly of the large aggregating type (50% - 85%), the large non-aggregating type (10% - 40%) and distinct small molecules (Heinegard 1981, Manicort 1986, Sampaio 1988, Carney 1988). The proteoglycans that contribute most significantly to the material properties of cartilage are the large, high molecular – weight monomers which consist of an extended protein core with several distinct regions (Hascall 1981, Heinegard 1984, Hassel 1986, Kuettner 1986). These large monomes typically contains some 80 – 100 chondroitin suphate chains, 30 keratan sulphate chains, 50 O-linked oligosaccharides and 5-10 N-linked oligosaccharides. The distribution of these carbohydrate substituents within distinct regions in the molecule is determined by the structure of the core protein, a peptide chain of about 2,000 amino acids. Even without the carbohydrate chains, this would be a large molecule; for comparison the length of the alpha-chains of the common collagens is about 1,000 amino acids.

Recent work has shown that the proteoglycan peptide chain is subdivided into at least three distinct regions (Hardingham 1986 A&B, Heinegard 1986, Oldberg 1987, Lohmander 1986): One, an amino terminal end of the protein core which has two globular domains that contain few or no glycosaminoglycan chains, but most of the N-linked oligosaccharides (Lohmander 1980). This amino-terminal globular domain of the core interacts specifically with hyaluronates and cartilage link protein in the formation of proteoglycan aggregrates. Since aggregration immobilises the proteoglycans in the matrix this region of core is of great functional importance. Two, an intermediate region of the protein contains a large part of the keratan sulphate chains and O-linked oligosaccharides; Three, the remaining carboxy-terminal domain of the core, which corresponds to about half of the protein, holds all the chondroitin sulphate chains, some keratan sulphate chains and O-linked oligosaccharides. In addition newly synthesised proteoglycans contain a globular protein domain at the carboxy-terminal end (Doege 1987, Oldberg 1987) which is devoid of glycosaminoglycans but may contain N-linked oligosaccharides (Figure 1.2).

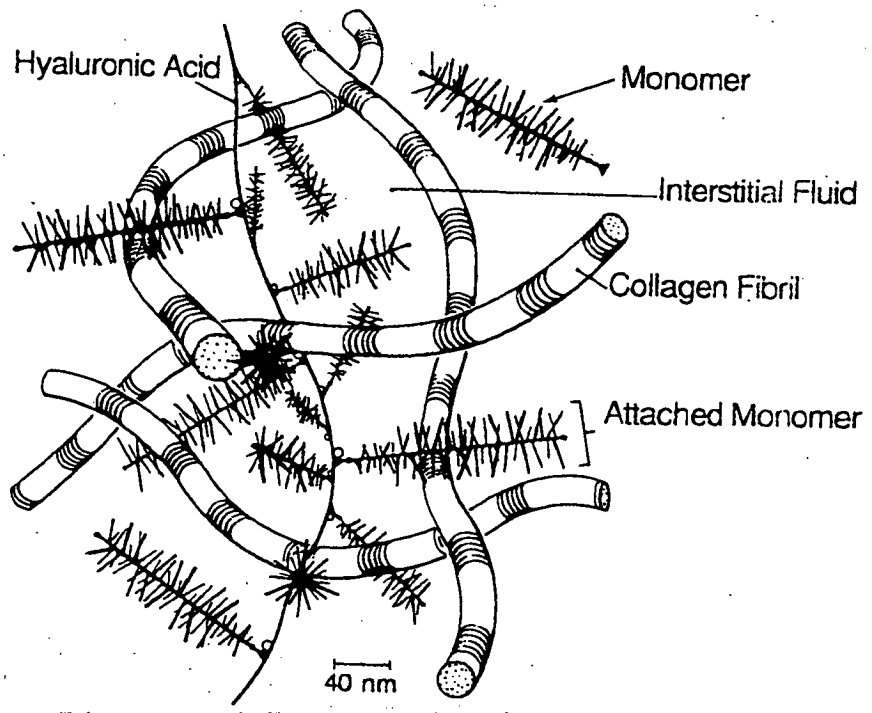


Figure 1-1 Diagrammatic Representation of 3D collagen mesh entrapping hydrated proteoglycans

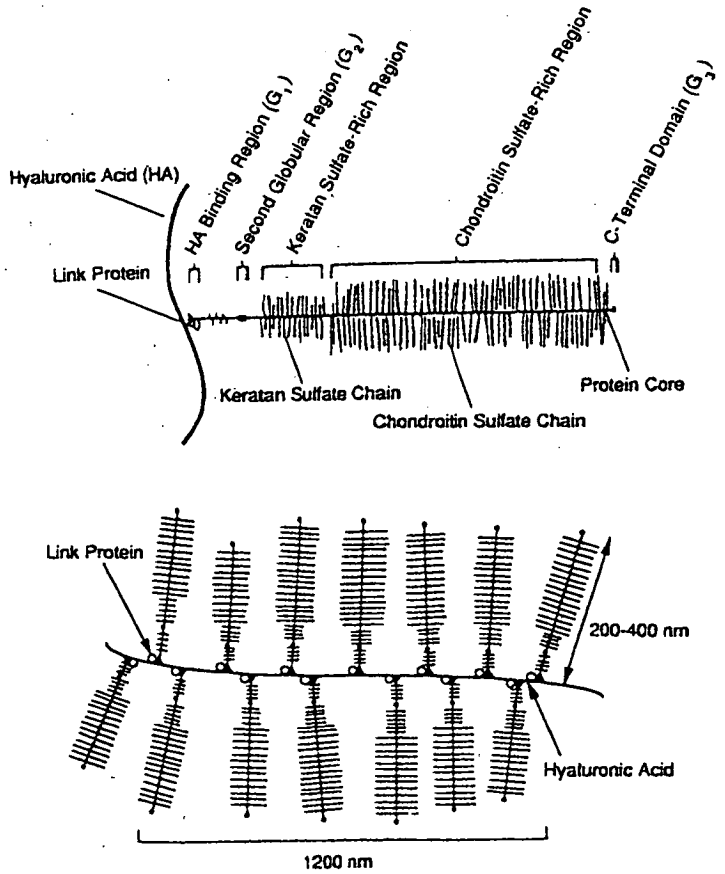


Figure 1-2 Diagrammatic Representation of an aggregating proteoglycan polymer

1.2.2.1 Glycosaminoglycans

Carbohydrates represent about 90% of the large cartilage proteoglycan, the principle glycosaminoglycan chains being chondroitin sulphate and keratan sulphate. The precise number of chains on a proteoglycan isolated from the matrix may vary from molecule to molecule, depending on the degree of carbohydrate substitution along the core protein. Chondroitin sulphate monomers consist of some one hundred chains per molecule. These chains consist of unbranched polymers of repeating disaccharides of N-acetylgalactosamine and glucuronic acid. Most of these hexosamines carry a sulphate group in the 4 or 6 position. These sulphate groups, together with the carboxyl groups, of the glucuronic acid provide fixed arrays of densely packed anionic charges along the glycosaminoglycan chains and are responsible for the hydrophilic and space-filling properties of the proteoglycan. Each chondroitin sulphate chain is attached to the core protein through a glycosidic bond between a serine in the peptide chain and zylose residue at the reducing end of the glycosaminoglycan chain (Muir 1958, Rodèn 1966). The linkage regions of the chondroitin sulphate chain to protein also contains two galactose residues and a glucuronic acid residue. Keratan sulphate monomers are largely located in the intermediate region of the core protein, and there are about 40 chains per molecule. Each chain consists of linear repeating disaccharides of an N-acetylglucosamine and galactose, where the hexosamine and sometimes the galactose carry a sulphate in the 6 position. The linkage of keratan sulphate to proteoglycan is via a glycosidic bond between serine or threonine and N-actelygalactosamine (Hopwood 1974) and this region of the chain is identical to the basic structure of the O-linked oligosaccharides (Lohmander 1980).

Significant variation occurs in the distribution of these proteoglycans and their glycosaminoglycans throughout the depth of the cartilage as reflected in differential histochemical staining (Christensen 1980, Yoshida 1982, Bayliss 1983, Ippolito 1983,). In addition, the biochemistry of these molecules changes with normal physiological ageing of the hyaline cartilage matrix (Sweet 1979, Mallinger 1987) for example, the percentage of keratan sulphate chains increase. The altered biophysical properties of articular cartilage, which occur in the development of osteoarthrosis, are again reflected in substantial changes in the biochemistry of the extracellular matrix (Mankin 1971B, Santer 1981, Teshima 1983, Bihari-Varga 1984,). These chondroitin and keratan sulphate chains account for about 80% of the glycosaminoglycan make up of the proteoglycans, the remaining 20% being made up of a number of lesser

glycosaminoglycan subtypes (Heinegard 1987). Included in this group are chains of heparan sulphate, heparin and dermatan sulphate (Rosenberg 1986). There is a more detailed discussion in relation to the sugar moieties that make up these chains and their synthesis in the chapter on lectins.

1.2.2.2 Oligosaccharides

Two types of oligosaccharides are commonly found in cartilage N-linked and O-linked. The 5-10 N-linked oligosaccharides are clustered in the amino terminal domain of the protein (Lohmander 1980). Amino acid sequence data indicates possible sites for these oligosaccharides in the carboxyterminal globular domain of the protein (Oldberg 1987). They are similar to the mannose-containing oligosaccharides that occur in many types of glycoprotein and are linked to the protein via an asparagine residue. The other type of oligosaccharide on the core protein, the O-linked is similar to the mucin-type of oligosaccharides binding glycoproteins. As in glycoproteins, there is considerable variation in size and structure within this family of oligosaccharides (Nilsson 1982)

1.2.2.3 Structural Role of Hyaluronate and Link Protein

The size, structural rigidity and complex molecular confirmation of normal proteoglycan aggregation promote proteoglycan-proteoglycan networking and proteoglycan-collagen interactions (Muir 1983). Inherent in this is the property of the dominant proteoglycans of cartilage matrix to interact with hyaluronate and link protein to form large aggregates, a functionally very crucial property of these molecules. These aggregrates contributing a number of properties to cartilaginous tissues including; compressive stiffness, Donnan osmotic pressure, regulation of tissue hydration, and shear stiffness. Aggregating interaction takes place in a region within the amino terminal domain of the protein core. The binding of this peptide region to hyaluronate is highly specific and requires a minimum decasaccharide of hyaluronate for optimal binding (Hardingham 1973, Hascall 1974). The stability of this binary complex of hyaluronates and proteoglycans is greatly increased by the presence of link protein. This protein interacts with both proteoglycan core protein and hyaluronate to form a ternary complex. The content of hyaluronate in cartilage is usually only about 1 – 2 % of that of the proteoglycan. Hyaluronate is a linear, unsulphated, polysaccharide of high molecular weight with alternating glucuronic acid and N-actelyglucosamines residues (Laurent 1986). Hyaluronate is unusual in that it is the only glucosaminoglycan, which

is not covalently bound to protein, even during synthesis in the chondrocyte (Caterson 1978,1979).

1.2.3 Noncollagenous Proteins and Glycoproteins

In addition to link protein there are a number of noncollagenous proteins and glycoproteins that contribute to the cartilage matrix structure. These include chondronectin (Hewitt 1982) and ankorin (Von der Mark 1986) which may help organise and stabilise the other matrix component molecules.

1.2.4 Matrix Water

Water is the most abundant component of articular cartilage accounting for 65% - 70% of the tissue weight (Linn 1965, Maroudas 1979, Mow 1984, Torzilli 1985 and 1988). A small percentage of this water is contained in the intracellular space, 30% is associated with the intrafibrillar space and the remainder is contained in the molecular "pore" the extracellular matrix. Inorganic salts, such as sodium, calcium, chloride, potassium, are dissolved in the tissue water (Linn 1965). Although these salts amount to less than 1% of the total weight, they play a significant role in tissue hydration, swelling, osmotic pressure and collagen pre-stress (Maroudas 1958, 1968, 1977). Water content is non-homogeneously distributed throughout the cartilage, decreasing in concentration from 80% at the surface to 65% in the deep zone (Torzilli 1985, 1988). Most of the water may be extruded from the tissue by applying a gentle pressure gradient across the tissue or by applying a compressive force to the solid matrix. Since there is a high frictional resistance to interstitial fluid flow through the porous-permeable solid matrix, the permeability of the tissue is low. This frictional resistance to fluid, combined with solid matrix stressors, balances the forces or fluid pressure applied to articular cartilage in vivo (Mow 1980, 1982).

1.2.5 Matrix Turnover

The matrix components of normal articular cartilage are continually but slowly turned over. The cartilage collagens have the slowest turnover rate being most resistant to proteolytic degradative enzyme attack. The protoeglycans, however, are much more susceptible to enzymatic degradation and have a much faster turnover rate. The proteoglycan "re-cycling" is controlled by a group of metalloproteinases secreted by the chondrocytes. The rate of break down is governed by the level of active

metalloproteinases and by the amount of tissue inhibitor of metalloproteinase (TIMP). Stromelysin is the enzyme predominantly responsible for the break down of proteoglycans (Galloway 1983) and collagenase is specific for the collagens (Murphy 1986). Proteoglycan degradation occurs through a major cleavage site at the protein core, which separates the G1 globular domain, involved in aggregation, from the glycosaminoglycan-bearing region (Tyler 1985, Ratcliffe 1986). The cleavage at these sites leaves that segment of the core protein that contains the glycosaminoglycan chains intact, therefore retaining the mechanical properties of the matrix. As the breakdown products are released from the cartilage they are replaced by newly synthesised components (Hardingham 1986A and 1986B). To replace the degraded matrix components the chondrocytes synthesise the complex and specific molecules at an appropriate rate controlling the composition structure and function of articular cartilage. Any disturbance of this process as occurs in ageing, or osteoarthrosis, leads to deterioration of the matrix weakening the structure of the cartilage and further contributing to the degradation of the tissue (McDevitt 1976, Eyre 1980, Muir 1983, Carney 1984).

1.2.6 Fibronectin, S100 protein, Chondronectin

In studies undertaken on animal and experimental models of osteoarthritis an increase in the glycoprotein fibronectin has been demonstrated (Miller 1984, Burton-Wurster 1986). This glycoprotein anatomically is noted as an amorphous material that accumulates around osteoarthritic chondrocytes, particularly in zone I (Rees 1987). Analytical studies show that the amount of fibronectin in the superficial zone is quite variable. Fibronectins have been found in both animal models of osteoarthrosis and human osteoarthrosis (Jones 1987, Burton-Wurster 1984 and 1985) and show a smiliar variable distribution.

S-100 protein is an acidic, calcium binding protein which was first demonstrated in the central nervous system, but more recently in a number of other tissues including chondrocytes (Takahashi 1981, Dranoff 1984, Vinores 1984, Pahlman 1986). Because the demonstrable immunohistochemical distribution of S-100 protein varies in cartilaginous tumours and evolving osteoarthrosis is a useful marker of these processes (Schmitt 1988, Okajima 1988). S-100 protein positivity has been shown to be present

in a wide variety of human cartilage types including foetal skull and visceral cartilage's along with chondrocytes cultured from chick embryos, in all cases the staining was intracellular (Stefansson 1982). Studies undertaken comparing cartilage in osteoarthrosis and rheumatoid arthritis have demonstrated that a similar spectrum of immunoreactivity for this protein is demonstrated in all chondrocytes at all levels (Mohr 1985). Studies specific to osteoarthrosis demonstrate an increase in S-100 protein expression in morphologically more severe disease and also within chondrocyte clones (Nakamura 1988, Rees 1988, Chen 1990). As chondrocytes originate from neural crest differentiation increased S-100 protein expression may represent regression to more "embryonic-like" chondrocyte activity, thus accounting for some of alterations in chondrocyte metabolic activity.

Chondronectin is a serum glycoprotein located pericellularly in cartilage. It is synthesised by chondrocytes and stimulates the attachment to type II collagen. Chondronectin bears oligosaccharides containing fuctose, mannose, N-acetyl glucosamine, sialic acid and galactose. It binds specifically to chondroitin sulphate rather than to other glycosaminoglycans. It is thought to organise the matrix close to the cell sulphate by forming complexes with cartilage chondroitin sulphate proteoglycans and type II collagen.

1.2.7 Component Interactions

Each of the major components of articular cartilage; proteoglycans, collagens and water has its own set of material properties and mechanical behaviour. The material properties of the tissue are governed by the complex interactions between solid and fluid phases and between components within each phase. For example, proteoglycan-proteoglycan interactions generate strong intramolecular and intermolecular charge-charge repulsive forces because of the closely spaced and negatively charged sulphate and carboxyl groups on the glycosaminoglycan chains (Maroudas 1968, and 1978, Mow 1984 and 1989, Hardingham 1987). These forces tend to expand and stiffen the proteoglycans entrapped in the collagen network. The negatively charged groups on the proteoglycans interact with the positively charged mobile cations in water of sodium and calcium to generate substantial osmotic swelling pressure (Maroudas 1979, Myers 1984 A&B). The magnitude of this swelling pressure is related to the density of the charged groups on the proteoglycans and the concentration of cations in the interstitial fluid.

The osmotic swelling pressure generated by proteoglycan-ion interactions is resisted and balanced by the tension developed in the collagen network surrounding the proteoglycans. Consequently, the collagen network is subjected to tensile stress even in the absence of externally applied loads. Thus, the interactions of proteoglycans with collagen fibrils, water, ions and with other proteoglycans contribute to the mechanical and material behaviour of the articular cartilage. This strongly cohesive nature of the collagen-proteoglycan extracellular matrix is maintained by interactions between these two macromolecules. The cohesiveness depends in part on the large proteoglycan aggregrates that promote entanglements that immobilise proteoglycans in the collagen network (Muir 1977, Nimni 1988, Mow 1989). In addition, mechanisms that effect protoglycan-aggregration, collagen cross-linking and proteoglycan-collagen interactions disrupt the cohesiveness of the solid phase and impair its ability to withstand internal swelling pressure and large, external applied loads. Disruption of the collagen cross-linking also diminishes the intrinsic tensile stiffness and strength of articular cartilage. It is these changes within the matrix of the cartilage which alter its biomechanical and histochemical staining properties (Broom 1988) in ageing and osteoarthrosis.

1.2.8 Cartilage Zones

Articular cartilage can be considered as a series of five zones based on the shape, number and arrangement of the cells and on the quantities, molecular composition and macromolecular organisation of the collagens and proteoglycans. This zoned division is convenient in analysing the extent of diseases and reflects the fundamental heterogeneity of articular cartilage so that the intrinsic mechanical and tinctorial properties of the different zones can be assessed (Figures 1.3 and 1.4).

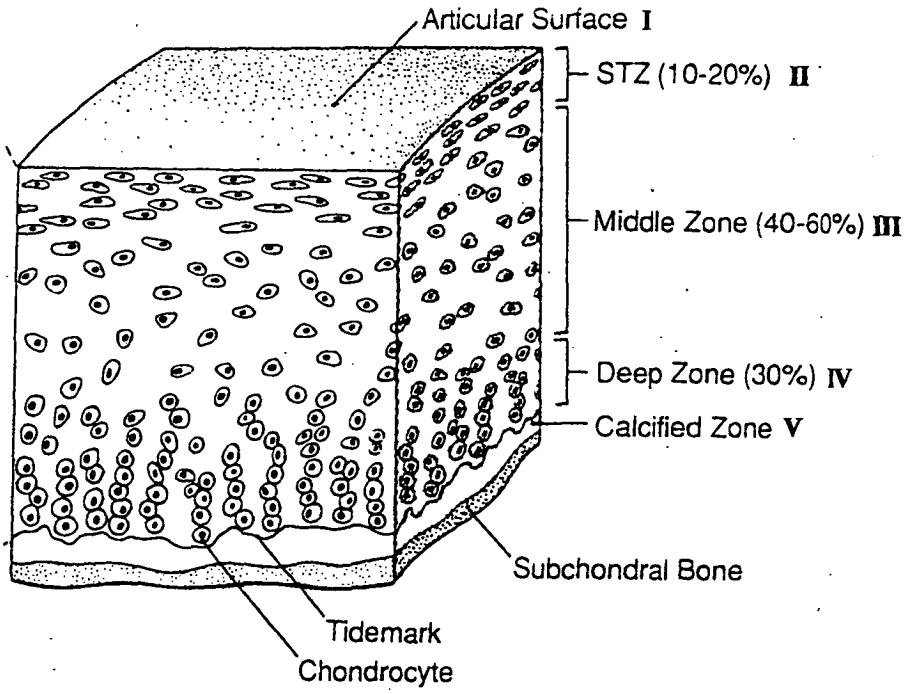


Figure 1-3 Diagrammatic Representations of Cartilage Zones

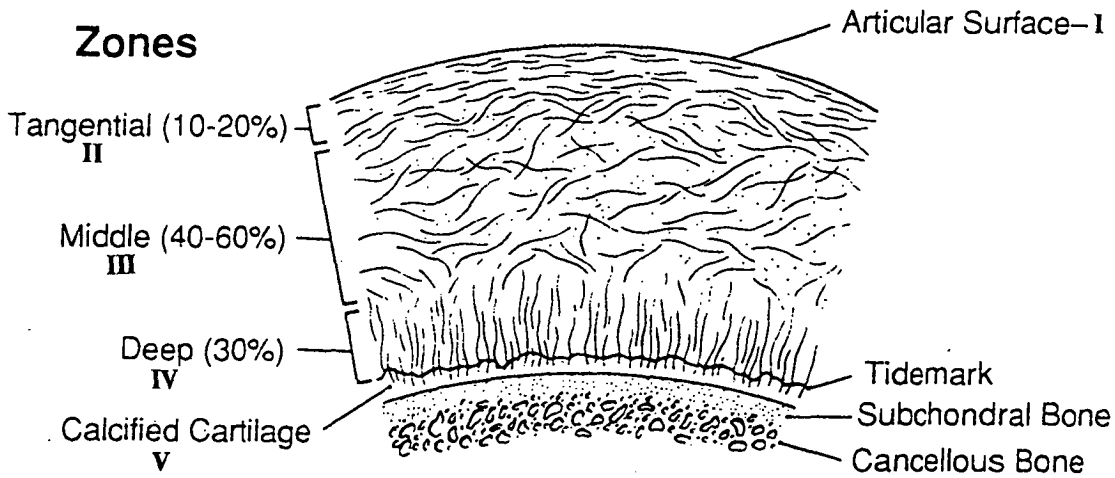


Figure 1-4 Diagrammatic Representation of Ultrastructure of Cartilage Collagen Network

1.2.8.1 Zone I

In an intact joint one can consider an interface sequence of *bone-cartilage-synovial fluid-cartilage-bone* that acts as a functional physiological continuum. Examined in a perpendicular light microscopic section, zone one is a dense layer of collagen arranged as orderly, interlacing fibre bundles lying tangential to the articular surface (Gardner 1983). The outer most layer is called the lamina splendens and represents the collagen-rich zone on the surface of the cartilage interfacing between the solid and liquid phases between cartilage and synovial fluid (Benninghoff 1925, MacConnaill 1951, Dunham 1988A). The chondrocytes of zone one are plate-like and they lie very close to the free disarticulated surface.

1.2.8.2 Zone II

The chondrocytes are ovoid or round. In the upper part of zone II they are single, but progressing more deeply an increasing number present as pairs within one chondron microenvironment (Poole 1987 and 1990). Pericellular matrix is more abundant than around zone I chondrons. The interterritorial matrix is comprised of a meshwork of collagen with fibres extending from various distances in three dimensions between the chondrons; it constitutes a porous, strong and elastic microskeleton, the interstices of which are occupied by proteoglycan macromolecules and water filled domains. The collagen fibres are perpendicularly orientated as opposed to the more horizontal orientation in zone I.

1.2.8.3 Zone III

The cells are widely dispersed, plump, ovoid or round and are not in pairs. The further they lie from the surface, the larger they appear. The territorial matrices contain collagen strands similar to that of Zone II and a high proportion of extracellular non-fibrous proteoglycan. The principle glycosaminoglycan is chondroitin sulphate that is more abundant that in Zone II, keratan sulphate is scanty.

1.2.8.4 Zone IV

The concentration of proteoglycan is high but varied, the cells are now seen sometimes in triplets or in places forming well-orientated vertical columns towards the "tidemark". Zone IV matrix dips into the zone V matrix as finger-like projections. As one passes into deeper parts of zone IV, the proportion of chondroitin sulphate decreased with a relative increase in keratan sulphate.

1.2.8.5 Zone V – Calcified Zone

Zone V is composed of calcified cartilage and is the deepest part of the articular cartilage separated from the non-calcified cartilage zone IV by the tidemark. Between this calcified cartilage zone and the underlying subchondral bone is a cement line. The chondrocytes of zone V exhibit properties akin to the underlying subchondral osteocytes and produce hydroxy-apetite crystals (Davis 1962,1969). Zone V cartilage varies in thickness but is remarkably constant in different joints in a single species and within different species (Meachim 1984A). This complex zone acts as a physiological and a mechanical interface that separates viscoelastic non-calcified articular cartilage from the underlying rigid bone. Its responses to ageing and osteoarthrosis are poorly understood yet this region is undoubtedly of physiological significance.

1.2.9 Cartilage Regions

A thin layer of pericellular matrix covers the surface of each chondrocyte and has intimate contact with the cell membrane. The pericellular matrix contains little or no fibrillar collagen being formed primarily of non-collagenous proteins, glycoproteins and proteoglycans. Enclosing the pericellular matrix is an envelope of territorial matrix, which contains fine collagen fibrils forming a basket-like meshwork around the cells. Where cells are widely separated, each has its own interterritorial matrix, but where cells like close together, they share a matrix. In most areas, the interterritorial matrix is considerably thicker than the territorial and pericellular matrix. The interterritorial matrix, which forms the largest matrix compartment, contains collagen fibrils having the largest diameter. Pericellular and interterritorial matrices serve the need of the chondrocytes by attaching the cell membranes to the matrix and creating an appropriate metabolic and mechanical environment for the cells. The interterritorial matrix is

primarily responsible for the mechanical properties of the tissue forming the largest overall region.

1.3 Tidemark

A clearly defined zone the tidemark is seen between the calcified and non-calcified cartilage interface. This line is said to be stained by a wide variety of dyes for proteins and lipids but not proteoglycan, is present only after decalcification and is biochemically undefined (Green 1970, Redler 1974, Spinelli 1976, Dmitrovsky 1978, Lane 1980, Broom 1982, Bullough 1983, Havelka 1984 and 1986, Oegema 1992,). Speculation concerning the composition of this line range from the presence of specific molecules to the non-specific binding of protein and lipids analogous to the binding of proteoglycan at mineralisation interfaces (Boskey 1976, 1980, Boyan-Salers 1980, Revell 1990). Collagen fibres in the deep layer of unmineralised cartilage that are perpendicular to the surface and parallel to each other become continuous through the zone of calcified cartilage and disrupted at the bone interface. As with the cement line seen in bone, no connections have yet been demonstrated between the bone and the zone of calcified cartilage. The tidemark normally forms a single line, however, re-duplication is seen both in ageing and early in the osteoarthrotic process along with other changes including vascular invasion (Hough 1974, Woods 1970, Havelka 1986, Hurrell 1935). Associated with this disruption is alteration in the thickness of the calcified cartilage that becomes thinned as the severity of osteoarthrosis increases (Muller-Gerbl 1987, Gilmore 1987, Meachim 1984B). Conjecture arises as to the functional interdependency between the subchondral bone and overlying non-calcified-calcified articular cartilage boundary. Some arguing that there are no physiological connections between the two and that nutritional pathways exist only from the synovial fluid to the hylaline cartilage (Greenwald 1969, Hodge 1969, Honner 1971, Ogata 1979). Much work remains to be done in relation to the three dimensional structures and pathophysiology of this unique zone. Some work in relation to the microanatomy was undertaken in this project.

1.4 Chondrocytes

The material properties of articular cartilage depend on the composition and structure of the extracellular matrix, and the synthesis and maintenance of this matrix is dependent

on the chondrocytes. During embryogenesis, articular cartilage forms from densely packed mesenchymal cells that differentiate into chondrocytes and synthesise the extracellular matrix. As the cartilage matures during skeletal growth, a large interterritorial matrix separates the cells. By maturation, these cells occupy only about 5% of the cartilage volume and lack physical cell to cell contact, except in Zone I. Despite their relatively sparsity, they maintain the cartilage matrix throughout life. Since cartilage lacks blood and lymphatic vessels, the survival and synthetic activity of the chondrocytes depends on the diffusion and transport of nutrients and metabolics throughout the matrix. The chondrocytes responds metabolically to exogenous and endogenous chemical factors in the matrix such as interleukins, growth factors and pharmaceutical agents (Tyler 1985, Ratcliffe 1986), matrix composition (Hardingham 1986B), mechanical loads (Tammi 1987, Gray 1988, Sah 1989) and hydrostatic changes (Mankin 1969, Kimura 1985, Hall 1989). The structure of the matrix is organised in such a way that loads are distributed evenly ensuring that the chondrocyte is protected from excess loads but are not isolated from their mechanical environment. Although the chondrocytes generally maintain a stable matrix, the response to some factors, such as interleukins or repetitive loads may lead to a change in matrix composition and eventually cartilage degradation.

1.5 Subchondral Bone Cells

Articular cartilage overlies the subchondral bone and the maintenance of the matrix and mechanical properties of this bone is dependent on the intrinsic cells of this region.

1.5.1 Osteoblasts

Osteoblasts line the external (periosteal) and internal (endosteal) surfaces of growing bone. The cells are elongated and are attenuated in section and flatter and ellipsoidal when viewed *enface*. Large, ovoid nucleus contain a single, conspicuous nucleolus. Delicate processes of fine cytoplasmic, fibrillar material are seen around cells (Bernard 1969, Boskey 1981, Boivin 1983, Bab 1984). These cells are intimately involved in modulating bone formation and react according to genetic programs. They manifest alkaline phosphatase and latent collagen activity. In particular they are involved in the production of type I collagen, and the many other glycoproteins and proteoglycans characteristic of mineralising tissues.

1.5.2 Osteocytes

Osteocytes are uninucleate cells that communicate with the vascular system, with osteoblasts on the surface, and with each other through a radiating, intricate network of canaliculae. Each osteocyte occupies a lacunar space in which its cell membrane generally appears to fit closely against the lacunar wall, although a gap is sometimes apparent which may or may not be a shrinkage artefact. The function of the osteocyte is integral in maintaining the mechanical and metabolic integrity of the matrix, osteocyte death being synonomous with bone death. The physiological function of the osteocyte is not fully understood, however, it is intimately involved in maintaining the bone matrix and interacts in a multi-functional manner with other bone cells such as osteoblasts and osteoclasts. The life cycle of the osteocyte is said to pass through four phases, formative, steady-state, reabsorbative and finally degenerative (Bonucci 1990).

1.5.3 Osteoclasts

The osteoclast is a large, multi-nucleated cell, characteristically found on ordinary endosteal surfaces, not to be confused with another multinucleated cell, a common inhabitant of the bone marrow, the megakaryocyte. On the basis of morphology it was originally thought the osteoclast was the principle agent of bone destruction, an idea no longer universally accepted (Marks 1983). Osteoclasts are intimately involved in bone resorbtion and bone remodelling and have a complex interdependency with the other main bone cells the osteocyte and osteoblast. Also defined is the chondroclast that is the osteoclasts cartilagenous counterpart and is seen "invading" uncalcified cartilage at the disrupted tidemark region in osteoarthosis. This cell's origins and functions are as yet poorly defined (Bonucci 1981).

1.6 Mechanical Properties of Cartilage

Articular cartilage has two primary properties in joints: (1) minimising and decreasing contact stresses through deformations that increase joint contact area (Mow 1984, A/B Ahmed 1983) and (2) contributing to lubrication of the joints through fluid efflux and redistribution of the surface within the tissues, allowing relative movement of opposing joint surfaces with minimal friction and wear (Mow 1968). When an external load is applied to a diarthrodial joint, such as the knee, a complex distribution of tensile, shear,

and compressive stresses is generated within the articular cartilage. When the load is removed, cartilage resorbs the fluid exudate, allowing the tissue to recover its initial dimensions in the unstressed state. These deformation and recovery mechanisms depend on interstitial fluid flow. Movement of interstitial fluid gives rise to the mechanical behaviours known as biphasic creep and stress-relaxation, which, in turn, enable articular cartilage to withstand the normally high and repetitive loadings on the joint. This visco-elastic characteristic defines the time-dependent property when subjected to a load or deformation. For example, when a constant stress is applied to the tissue, the deformation increases with time or "creeps" until equilibrium is reached. Similarly, when the tissue is deformed and held at a constant strain, the stress rises to a peak and slowly relaxes to equilibrium (Edwards 1967).

In materials such as polymethylmethacrylate, such visco-elastic behaviour arises from the relative motion of long chain molecules that generate marked inter-molecular friction causing energy dissipation (Ferry 1970), however, in biphasic material such as cartilage, both fluid exudation and solid deformation give rise to the observed visco-elastic behaviour. Firstly through the intrinsic visco-elasticity of the solid phase, arising from the motion of polymeric chains such as proteoglycans and collagen, during deformation. Secondly, in response to an applied load or pressure gradient, frictional interactions arising from interstitial fluid flow through the pores of the solid matrix and contribute to the time-dependent visco-elastic nature of articular cartilage (Mow 1980, 1984). These two deformational mechanisms help explain the behaviour of articular cartilage or in fact any other hydrated soft tissue. In the laboratory it is possible to create simplified states of stress or deformation in order to characterise the tissue and illustrate the behaviour of articular cartilage in vivo. Through these analyses, it is possible to understand how the functioning of compromised cartilage promotes the progression of osteoarthrosis or, conversely, how altered joint loading impairs cartilage function. The results of such controlled experiments can assist in showing how altered mechanical behaviour correlates to altered structure in the collagen-proteoglycan matrix interactions, as demonstrated by micromechanical studies (Roberts 1996).

1.7 Ageing of Cartilage

Maturation of hyaline articular cartilage is complete at a relatively early stage in life thereafter ageing changes advance remorselessly. Senescence is considered a natural physiological event that effects all human tissues and their subcomponents; cells and

extracellular matrix (Young 1974, Bittles 1986). There are many pathological similarities between the changes seen in ageing articular cartilage and in joints with early osteoarthrosis. Ageing cartilage is prone to the pathologies which predispose to osteoarthrosis (Freeman 1972, 1979) and therefore an understanding of the "natural ageing process" is important in defining the overall osteoarthrotic process (Maroudas 1990, Gardner 1991, Mow 1987).

1.7.1 Ageing of Cells

Studies have suggested that ageing of chondrocytes leads to adverse change in all parameters of cartilage growth including replication and biochemical functions in the production of collagens and extracellular matrix (Vignon 1976, Mort 1983,Roughley 1987). In vitro monolayer cultural studies strongly suggest articular cartilage chondrocytes from old rabbits show a decrease in functional ability (Dominice 1986).

1.7.2 Ageing of Extracellular Matrix

Many changes in the proteoglycans have been shown with ageing (Elliott 1979, Labat-Robert 1988, Venn 1978). The total quantity of proteoglycan in ageing human cartilage changes very little (Bayliss 1978) however, there are important alterations in synthesis, largely affecting glycosaminoglycans. The chondroitin sulphates diminish in relative amount and in the deep zones are replaced to an increasing degree by keratan sulphate. There is ultimately a depletion of the large glycosaminoglycan-rich-proteoglycans, essential for normal cartilage behaviour, a progessive sequence that can contribute to the destructive changes of osteoarthrosis (Buckwalter 1985). The structure of proteoglycan subunits alter, there is a decrease in the number and size of the chrondoitin sulphate chains and an increase in 6-relative to 4-sulphation. An increase in the number and size of keratan sulphate chains is recognised, together with a decrease in the number of O-linked oligosaccharide chains per core protein, the amino acid composition of which also alters. The hyaluronate-binding region is unaltered. Overall there is a predominance of smaller, less glycosylated proteoglycans subunits (Roughley 1986, Theocharis 1985, Elliott 1979). Various mechanisms have been postulated for these changes including: one variation in core protein gene expression, two varied activity of post-transitional enzymes, and three modification of proteoglycans by proteolysis after the secretion of proteolytic enzymes (Roughley 1986). It is probable

that all these three mechanisms are acting together to provide those changes seen in ageing.

1.7.3 Ageing of Collagens

There are characteristic changes in the size and organisation of articular cartilage collagen fibres as age advances (Verzar 1964). There is an overall increase in fibre diameter attributable to the aggregation together of individual fibres (Mallinger 1988). The formation of these thicker fibres with advancing age is a result both of collagen fibre remodelling and of alterations in the proteoglycan size and composition. A small change in the proportion of collagen types in ageing has been suggested, there is a slight increase in type II collagen with advancing age (Nemeth-Csoka 1983), and a slight decrease in type XII collagen. There is also a known increase in minor collagens such as type X collagen in degenerative joint disease as this is associated with reparative changes in articular cartilage. As in osteoarthrosis, there is a change in collagen cross-links which subsequently affects proteoglycan stereochemistry (Light 1979). Alteration in cross-links has been identified to be associated with the existence of elevated protease activity and the presence of diminished levels of protease inhibitors. Studies on the non-weight-bearing and the weight-bearing parts of tibial condylar cartilage reveals a rise in neutral metallo-enzyme activity in older cartilage associated macroscopically with surface roughening (Martel-Pelletier 1987A/B). Protease enzyme activity is higher in non-weight-bearing rather than it is in weight-bearing areas of the joint. In both the aforementioned groups enzyme activity is higher in the superficial zones than it is in the deeper zones.

1.7.4 Ageing and Cellularity

The cellularity of hyaline articular cartilage is high in the foetal period and declines with time being particularly marked in the elderly (Stockwell 1979A/B). This alteration in cellularity is variable between joints and also between different zones and regions. Many of the original observations of articular cartilage were conducted on dehydrated tissue prepared as stained paraffin embedded sections (Stockwell 1967). The problems of shrinkage and dehydration were overcome using frozen human cartilage blocks and allowed for more accurate morphometric assessment of the cellular content of the cartilage (Quintero 1984A & B). Studies on femoral head cartilage cellularity show that the most significant changes occur in zone I and II and that there is a cellular decrease

of up to 35% between the fourth and ninth decades of life (Vignon 1976). With increasing age, there are an increased number of cell clusters (clones) and a decreased number of cells in the weight and non-weight bearing region of the femoral condyles (Mitrovic 1983&1987, Quintero 1984A & B). These studies also showed fibrillated cartilage has a lower cell density than non-fibrillated and that grossly normal cartilage from joints with osteoarthrosis displayed a lower cell density than cartilage from normal joints. Chondrocyte loss may occur through direct damage due to interleukins or repetitive trauma leading to occasionally seen light microscopic changes such as pyknosis and karryohexis. In addition, cell drop out by programmed cell death (apoptosis) may be a part of the normal ageing process. These patterns of cell loss are not well researched in articular cartilage and as the chondrocyte is the matrices biochemical "powerhouse" a further understanding of the processes is needed to define the pathophysiology of ageing in cartilage (Mitrovic 1983 & 1987, Quintero 1984 A & B).

1.7.5 Ageing and Water Content

With advancing age, the water content of articular cartilage changes. In full thickness femoral head sections varying from approximately 76% at 15 years of age to 69% at 70 years of age (Venn 1977 & 1978). These decreases were more pronounced in the deeper zones over the superficial zones. An increase of fixed charge identity is observed in parallel with a reduction in water content reflected in decreased staining intensity using tinctorial stains for proteoglycans, such as toluidine blue and alcian blue.

1.7.6 Ageing and Surface and Structural Changes

Articular cartilage surfaces alter with age showing deterioration with time (Freeman 1972, Gardner 1987). Surface scanning electronmicroscopy (SEM) techniques used on exposed, moist, non-loaded adult articular hyaline cartilage show that the 3-D surface pattern mirrors underlying Zone I chondrocytes which decline in number with age. (Longmore 1978). This change in pattern reflects an alteration in the molecular composition of the extracellular matrix and precedes a series of alterations in colour, roughness and matrix organisation demonstrable by SEM (Gattone 1982).
The extent of the surface roughening of cartilage has been examined in detail by a number of authors, some of whom used Indian ink to enhance these changes (Freeman 1969, 1972, 1975 A/B, 1979, Meachim 1972A/B, 1975A/B, 1976, 1982). The surface

topography and morphology of the shoulder, hip, patella, femur, tibia and ankle joints were demonstrated to show areas of minimal fibrillation, overt fibrillation, bone exposure and osteophytosis. Also anatomical features such as horizontal splitting of tidemark, cartilage thinning, peripheral fibrosis and loss of subchondral bone were noted (Meachim 1972A/B, 1975, 1978, 1982).

Studies on the hip joint were set up to test the hypothesis that limited degenerative changes of the hip joint are related to ageing and that independently there are progressive degenerative changes that can be regarded as osteoarthrosis (Byers 1970, 1974, 1976 and 1977). These studies demonstrated statistically that there are two independent types of cartilage pathology, an ageing process and an osteoarthrotic process. Ageing changes were less severe and more focal, related to weight-bearing areas as opposed to osteoarthrotic changes that were more generalised with more significant pathological changes. In ageing they defined gross lesions of "limited progression" which microscopically exhibited fibrillation, splitting, a fibrous surface layer, cellular resorption and ossification. In osteoarthrosis "progressive lesions" were identified solely as fibrillation of the cartilage with associated osteophyte formation and cyst formation in underlying bone. These studies were broad based, limited by relatively small cohorts and were unable to demonstrate that the changes of ageing were not capable of progressing to osteoarthrosis. A small study on the human knee joint showed that the frequency and severity of cartilage lesions increased with age and that femoral lesions were more common than patella lesions, both being more common and severe in women (Mitrovic 1987).

All these studies show clear evidence that pathological changes may occur in joints from a relatively early age in life and advance with ageing. Other factors may lead to more rapid progression of these changes and/or development of osteoarthrosis. The aetiopathogenesis of ageing and osteoarthrosis are undoubtedly interwined and therefore a clear understanding of both is important in cartilage pathology.

1.7.7 Ageing and light microscopic changes

In the "early" phases of ageing the surface of the joint may appear normal (Lagier 1971, 1976, Freeman 1975, 1979), however, microscopic studies show a decrease in the number of zone I chondrocytes and biomechanical studies an alteration in cartilage

biomechanics implying submicroscopic changes (Roberts 1996). As ageing "advances" Indian ink staining showed minimal superficial fibrillation and tangential splitting of the superficial cartilage. Later as the fibrillation "progresses" there may be deeper splits running both vertically and parallel to the surface. Horizontal splitting may also be seen at the tidemark (Meachim 1978), however, this may represent an artefact in sectioning of fixed cartilage. Altered metachromasia of articular cartilage matrix is seen (Mallinger 1987) as an increased hetereogencity in intensity of staining in the different regions and zones of articular cartilage with glycosaminoglycans stains. This alteration is different to the systematic loss of staining in the superficial zones seen in osteoarthrotic cartilage (Christensen 1980).

1.8 Osteoarthrosis

Osteoarthrosis is the commonest form of arthritis (Creamer 1997) and is a major cause of morbidity in the elderly as well as being a burden on health-care services. The understanding of the pathogenesis of osteoarthrosis has advanced significantly over the past 15 years and although the precise molecular cause of osteoarthrosis remains to be elucidated, it is known that genetic, environmental, metabolic and biochemical factors can contribute to its pathogenesis. It has been difficult to encapsulate osteoarthrosis in a simple definition, however, in 1975 a consensus statement was put forward by the American Academy of Orthopaedic Surgeons (Keuttner 1975). "Osteoarthritic diseases are the result of both mechanical and biologic events that destabilise the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix and subchondral bone. Although they may be initiated by multiple factors including genetic, developmental, metabolic, and traumatic osteoarthritic diseases involve all of the tissues of the diarthroidal joint. Ultimately, osteoarthritic diseases are manifested by morphologic, biochemical, molecular and biomechanical changes of both cells and matrix which can lead to a softening, fibrillation, ulceration, loss of cartilage, sclerosis and eburnation of subchrondral bone, osteophytes and subchondral osteocytes. When clinically evident, osteoarthritic diseases are characterised by joint pain, tenderness, limitation of movement, crepitus, occasional diffusion and variable degrees of inflammation without systemic effects".

1.8.1 Theories of osteoarthrosis

For many years osteoarthrosis was considered to be the result of a simple consequence of ageing and cartilage degeneration and the common diagnostic category of degenerative joint disease was applied to such cases. There are quite characteristic ageing processes that occur in articular cartilage and this label of degenerative joint disease is a misnomer (Creamer 1997). The contemporary view is that osteoarthrosis is a disease which results from active, multi-factorial processes that affect common pathways of change in cartilage metabolism ultimately culminating in hyaline articular cartilage degeneration. Osteoarthrosis may thus be viewed as a disease in which the normal balance between cartilage synthetic processes and degradation is disrupted, often with an accompanying inflammatory component. The precise temporal relationships of these changes and the factors that cause them remain unclear. It is thought that the observable histopathological features of osteoarthrosis must be preceded by metalobic and biochemical changes at the cellular and subcellular levels (Kraus 1997). Studies into the evolution of osteoarthrosis in humans is very limited by both ethical and practical considerations. The protracted time course of development of the disease is an additional, compromising factor. Therefore, much of the work on the understanding of the evolution of osteoarthrosis has been from animal models of osteoarthritis using both naturally occurring and induced disease in rabbits, dogs, mice, guinea pigs and other animals (Bendele 1989, Jurvelin 1985, Walton 1977&1979, Colombo 1983, Kessler 1986). Other authors have undertaken extensive studies looking at specific biochemical issues using cell cultures of chondrocytes (Dunham 1988A/B, 1990). Our understanding of osteoarthrosis is expanding and there remains much research to do before effective therapeutic regimes can be established to assist in controlling this disease (Bland 1983, Poole 1986).

1.8.1.1 Enzymatic cartilage destruction in osteoarthritis

The breakdown of cartilage in osteoarthrosis has been the subject of many studies over the last 30 years. Much of the work has described the structures and properties of the various enzymes that may be responsible for this degradation as well as natural and synthetic inhibitors of these proteinonases. Detailed analyses of the components of cartilage are focused on the large chondroitin sulphate and keratan sulphate-rich proteoglycans that aggregate with hyaluronic acid. Recently, there has been progress in understanding cartilage breakdown in human articular cartilage, in health and disease and the enzymatic and regulatory mechanisms involved in these in this process.

Collagen breakdown: Initial cleavage of cartilage matrix molecules involving collagens, is extracellular and is mediated primarily by proteinases. Degradation products may be further digested by proteinases and glycosidases within the lysosomes of chondrocytes or in remote sites such as the liver. The proteinase families found in cartilage matrix synthesised by chondrocytes include; collagenase, gelatinase, stromelysin, other acid metalloproteases, elastase, cathepsin G, B, L, D and kallikrein. The metalloproteinases are characterised by a requirement for Zn^{2+} in the active site, where it is probably retained by two histidines that are five amino acids apart in mammalian collagenase (Whitham 1986). The best known of these metallo-enzymes is collagenase and is the only enzyme capable of cleaving the triple helix of type II collagen. It cannot, however, cleave collagen types IX and XI (Gross 1980, Gadher 1988), the latter collagen being cleaved by the metallo-enzyme gelatinase (Murphy 1981, 1985). There is a complex interdependency on the degradative properties of these metallo-enzymes and the tissue inhibitor of metalloproteinases (TIMP) (Cawston 1981) inhibits them. The serine based enzymes, elastase and the cathepsins have the ability to cleave type II collagen and therefore destroy the tensile properties of cartilage by breaking down its three dimensional matrix (Starkey 1997, Bader 1981). In addition, these enzymes are able to cleave types IX and XI collagen at sites other than the metallo-enzymes (Eyre 1987, Van der Rest 1988, Mendler 1989). Stromelysin has the ability to cause degradation of cartilage proteoglycan aggregrates as it cleaves off individual proteoglycans (Campbell 1986, Azzo 1986, Clark 1987).

Proteoglycan monomers, which contain chondroitin sulphate and keratan sulphate are composed of three globular domains. At the N-terminal is G1 or the hyaluronic-acid-binding region; close to it, but toward the C-terminus is G2. Between G2 and a third globular domain, G3, at the C-terminus is the glycosaminoglycan attachment region (Doege 1987). Keratan sulphate is concentrated mainly on the C-terminal side of G2. Most of the chondroitin sulphate resides between the keratan sulphate and G3. Proteoglycan monomers form high-molecular-weight aggregrates with the hyaluronic-acid-binding region. These are stabilised by a molecule called link protein that binds to G1. Both G1 and link protein attach specifically to hyaluronic acid. When proteoglycan aggregates are cleaved by stromelysin, the primary cleavage appears to be between G1 and G2, producing a G1-hyaluronic-acid-binding-region domain. (Nguyen 1989) Link protein is also cleaved, producing LP3, the smallest of the three link proteins observed in adult human articular cartilage (Roughley 1982). Microsequencing

analysis has revealed that the cleavage of link protein by stromelysin is between histidine and isoleucine. The cleavage product LP3 progressively accumulates in situ in human articular cartilage as it ages (Mort 1983), as does the hyaluronic-acid-binding region of the G1 domain (Roughley 1985, Doege 1986).

The postulated In Vivo interactions of these proteinases and their inhibitors are shown in Figure 1.5. It is apparent that a specific activator regulates each proteinase and inhibitor and that there is a fine balance between activated proteinase and inhibitor. Any disturbance in this balance results in net proteinase activity or inhibition. Many of the other mediators of the inflammatory process are known to interact with TIMP. Interleukin-1, fibroblast growth factor and epidermal growth factors all stimulate synthesis of proteinases with the production of TIMP (Edwards 1987, Overall 1989, Murphy 1986). By contrast, transforming growth factor beta inhibits transcription of the proteinases but stimulates that of TIMP (Edwards 1987). The anatomical sites of initial action of these enzymes and other chemical mediators have been examined.

Morphological studies have revealed that swelling and disruption of collagen fibrils and reductions in diameter may occur at and below the articular surface early in the osteoarthrotic disease process (Poole 1986). Reduction in fibril diameter is seen in the mid-zone, and fibril disruption has been seen in mid-zone perilacunar sites (Pelletier 1983A/B, Orford 1983). In human cartilage, collagen loss is seen mainly in the middle and deep zones (Venn 1977). A new immunohistochemical method, employing antibodies that recognise only "unwound type II collagen alpha-chains" has been used to demonstrate that in osteoarthrotic human cartilage, collagen degradation takes place mainly at the articular surface, in the upper and middle zones and in pericellular sites (Dodge 1989). The reason for collagen degradation at these sites is not known, but it may occur in response to mechanical changes, which in turn, lead to changes in the loading of chondrocytes, interfering with biosynthesis and increasing matrix turnover. Although it has not been clearly established that mechanical loads can significantly increase or decrease matrix synthesis, particularly cyclic loading, little work has been done to investigate the effects of loading on degradation (Palmoski 1984, Van Kampen 1985). Mechanical forces acting upon chondrocytes may lead to significant changes in matrix turnover inhibiting biochemical synthesis and also directly cause degradation, therefore these affects acting together may magnify the damage occurring. As the disease becomes established, the effects of cytokines, such as interleukin-1, released

from synovial cells and possibly from polymorphonuclear leukocytes, probably enhance degradative trends. In addition, they lead to reductions in matrix synthesis.

Proteoglycan breakdown: As cartilage is progressively degraded, changes occur in the cartilage matrix. There is little or no loss of proteoglycan in human cartilage, but synthesis is often increased (Poole 1986). Histochemical studies of proteoglycans have revealed significant changes, with local loss at the surface. Immunochemical studies of the different proteoglycan populations have revealed that, compared with normal femoral condylar cartilage, osteoarthritic cartilage contain similar numbers of the larger proteoglycans (rich in chondroitin and keratan sulphate) and the smaller proteoglycans (rich in keratan sulphate and poor in chondroitin sulphate) (Rizkalla 1989). Although there is no evidence of increased degradation, foetal-like proteoglycans are synthesised, replacing those, which were lost. These are then degraded as part of the process of matrix turnover. In rheumatoid arthritis, collagen degradation is initially observed mainly at the articular surface at the edge of joint surface at the link-zone with bone and maybe related to the production of interleukin-1 from subchondral inflammatory tissue (Dodge 1989). Thus, in terms of matrix degradation, the features of osteoarthrosis differ from rheumatoid arthritis, although the proteinases involved are probably the same. The pathophysiology of matrix turnover and its regulation requires special attention in osteoarthrosis.

1.8.1.2 The role of trauma in osteoarthrosis

Another factor in the development of osteoarthrosis is the role of trauma either through direct damage to the cartilage or indirectly through damage to bone ligaments and synovial tissues. Repetitive-impact loads initiate direct cartilage degradation through the generation of high internal stresses, causing acute damage to cartilage matrix. The development of clinical signs may take many years to develop, however, such injuries may result in progressive cartilage degeneration as repair mechanisms fail to work. Such biomechanical failure of cartilage requires high tensile or sheer strain (Armstrong 1982, Radin 1986). Due to the unique visco-elastic properties of cartilage, large compressive stresses, slowly applied, rarely initiate cartilage degeneration. Whereas with rapidly applied loads as the articular cartilage and subchondral bone are visco-elastic and become stiffer and therefore deform less (Radin 1971, 1978, Repo 1977) stress relaxation cannot occur. Therefore high tensile and shear strains are generated at the osteochondral junction and at the margin of the joint (Armstrong 1985, 1986) and

result in degeneration. As water is not allowed to flow away from the high pressure areas to minimise the "shock loading" of the cartilage the reduced deformation decreases the contact area, further increasing stresses in the matrix (Askew 1978). The more rapid the impact, the larger the tensile and sheer stressors and the greater the probability that fibrillation will be initiated (Radin 1985). These pathological processes set up a sequence of tissue damage and repair and also lead to "a non-progressive cartilage softening" with or without fibrillation (Meachim 1975&1985, Mankin 1982, Radin 1984A/B). Arthroscopy performed at this stage will show a normal appearing yet softened joint surface with poor "visco-elastic" response on gentle indentation with a probe (personal observations). These observations probably reflect the earliest changes seen in otherwise visibly normal articular cartilage. Microscopy shows that chondrocyte death may occur in superficial cartilage loss or adjacent to vertical fissures, the fissure may spare cells in other layers showing the variability of this pathological process (Finlay 1978A/B).

High loading rates create high surface tensile strains that may cause breakage of the collagen fibres in the superficial tangential layers of cartilage (Askew 1978, Roth 1980). These changes may alter the biomechanical properties of the cartilage, however, this submicroscopic surface damage does not necessarily progress onto osteoarthrosis (Thompson 1975, Byers 1970). Although there may not be any disruption of the superficial layers significant sheer strains are generated in deeper cartilage layers (Burstein 1968, Hayes 1972). At a given load, the magnitude of the sheer stress is directly proportioned to cartilage thickness, increasing the potential for damage in areas with thick cartilage that may explain why impact damages patellae more than femoral cartilage (Donohue 1983). These high sheer stresses cause splitting between the layers of bone and cartilage (stress fractures), creating local stress concentrations that can lead to degeneration at the cartilage base without disruption of the tangential layer at the articular surface (Imai 1989, Goodfellow 1976, Radin 1985, Freeman 1975, Spirit 1986). This resultant basal disruption removes some of the constraints of cartilage deformation in the radial direction and damages the collagen fibrillar network, leading to progressive osteoarthrotic change (Finlay 1978B). Subsequent re-alignment of the normally pseudorandomly arranged fibres is seen by examination under polarised light as "crimping" of collagen, distinct from the smoother texture of non-traumatised cartilage (Broom 1980, 1983, 1986, 1988).

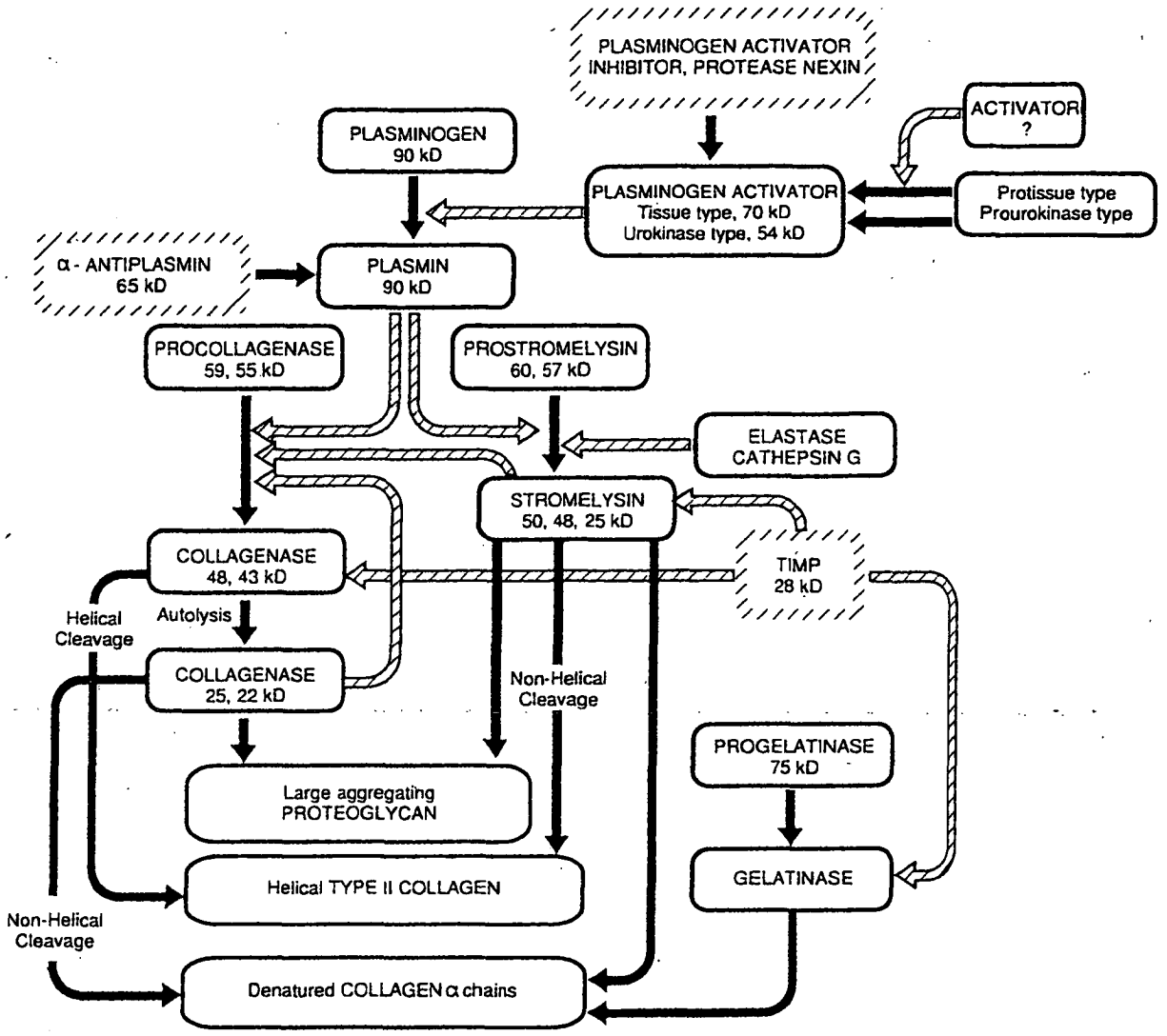


Figure 1-5 Metalloprotease interactions regulating enzymatic degradation of collagen

The temporal relationships are unclear, but matrix damage can precipitate compromised chondrocyte viability, loss of proteoglycans, or changes in hydration (Maroudas 1973, Altman 1984). The cellular and biochemical changes after impact loading are almost certainly reparative processes thus are secondary to a primary initiating event, however, once initiated this may be self-perpetuating and degradative (Thompson 1975, Radin 1984). For example, chondrocyte cloning after subfracture impact loading is rarely observed (Yang 1989, Radin 1984), however, chondrocyte cloning is a feature of other models of osteoarthritis that do not involve impact loads (Lukoschek 1988). Increased peri-cellular staining signalling enhanced proteoglycan synthesis is not observed until four – six weeks after single or repetitive impact loads (Yang 1989). The macro-molecular properties of cartilage proteoglycans following several weeks of impact loading remain unaltered even though cartilage matrix is lost, suggesting that the proteoglycan molecule is not responsible for this loss (Yang 1989). There are alterations in proteoglycan synthesis demonstrated as an increase in hexuronic acid after a single episode of blunt trauma (Donohue 1983). Therefore it seems most probable that the physiological protective mechanism is a reduction in the proteoglycan degradation (Mankin 1971A, Thompson 1979) and that these physiological insults result in an altered relationship between collagen and proteoglycans and presumably in the interdependency between cartilage proteases and their respective inhibitors.

Besides an insult to articular cartilage, trauma may lead to inflammation of pericapsular and synovial structures that may play a role in the initiation of osteoarthrosis. It has been suggested that chondrolytic enzymes play some role in the initiation of osteoarthrosis from trauma (Schwartz 1974, Ehrlich 1978, Pelletier 1985,1987A/B). Two possible mechanisms are acute synovitis, promoting cartilage degradation enzymatically (Lukoschek 1986, 1988) and secondary synovitis caused by debris from the destruction of the cartilage matrix (Chrisman 1969, George 1968). However, chondrolytic enzymes affect mainly the proteoglycans, not the collagen, and the role of such enzymes in the development of osteoarthrosis may be exaggerated. It is not necessary to implicate an inflammatory process to explain cartilage fibrillation following impact loading. Significant synovial inflammation does not occur for at least eight weeks after the start of repetitive impact loading, applied daily, or may never occur (Radin 1984, Lukoschek 1986). Sub-synovial fibrosis and hyperplasia are observed following impact loading (Lukoschek 1986) but hyperplasia occurs without evidence of significant inflammation. In relation to the development of OA

due to trauma exactly what role the para-articular soft tissues play in the process is not yet fully elucidated.

1.8.1.3 Primary and secondary osteoarthrosis

The two previous sections have considered the two theories that are considered to form common pathways in the development of osteoarthrosis in human joints. Myriads of ways of classifying osteoarthrosis exist and it is best to consider aetiology to be primary or secondary. The former may be the response to complex genetic social and epidemiological differences in population groups prone to develop this disease process. The latter is the result of a wide variety of lifestyle, disease related and environmental factors including; mechanical and traumatic, infective, immunological, inflammatory, endrocine and metabolic (Brandt 1981, Freeman 1972, Gardner 1980, Howell 1975, 1979, Sokoloff 1969 & 1979, Steinmann 1992).

1.8.1.4 Hereditary mechanisms in osteoarthrosis

Inheritance predisposes to a number of variants of secondary osteoarthrosis. For example, in hip dysplasia and the rare chondrodystrophies the inherited defect in chondro-osseous growth creates a series of anatomical defects that alter joint shape and congruity. The alterations in biomechanics ultimately leading to an alteration in the extracellular matrix. In idiopathic osteoarthrosis restricted to a few joints there is limited evidence to suggest that genetic factors are frequently involved. Some studies suggest that there could be a heritable component in disease susceptibility in some categories of these patients, for example, a defect in blood group O donors has been shown in patients undergoing hip arthroplasty for idiopathic osteoarthrosis (Lourie 1983). However, in primary generalised osteoarthrosis, the operation of multiple genetic factors is almost certain, although no subgroup associated with any particular HLA genotype is known (Lawrence 1962, Kellgren 1963). Overall, the evidence suggesting a genetic basis for common forms of osteoarthritis in man is compelling. The relationship between osteoarthrosis and genotype has been clearly demonstrated in many strains of in-bred mice. For example, the STRN1, C57/B1 and the STR/ORT strains are all high susceptible to osteoarthrosis (Sokoloff 1956, Dunham 1990, Walton 1979).

1.8.1.5 Growth factors in osteoarthrosis

In normal mature adult articular cartilage mitotic figures are not seen. However, the presence of clusters or clones of cells in osteoarthrosis demonstrates that these cells are capable of division and new DNA synthesis. In addition, experimentation has shown that these cells exhibit abnormal metabolic behaviour with abnormalities in their newly synthesised proteoglycans (Kresina 1986, Martel-Pelletier 1987, Williams 1988). Osteoarthrosis may be a local expression of disordered chondrocyte growth or endocrine regulation. Within cartilage there is a sensitive system that regulates collagen and proteoglycan secretion by feedback control. After maturity, chondrocyte metabolism, synthesis and secretory activity continue to be regulated by growth hormone, which influence cartilage cells through somatomedins produced by the liver (Harris 1980). Although somatomedins can act directly upon chondrocytes, for example by stimulating glycosaminoglycan synthesis (Kemp 1980), there is no direct evidence that abnormal chondrocyte metabolism and growth is attributed to alterations in somatomedins synthesis release or action. However, the are undoubtedly as yet not understood regulatory mechanisms which allow for "switching on" or "off" of chondrocyte replication.

1.8.1.6 Biochemical disorders in osteoarthrosis

Altered biomechanics may lead to cartilage degradation as reflected by alterations in the molecular and metabolic attributes of the cartilage. The antithesis of this may be that subtle primary alterations in joint metabolic activity may predispose to osteoarthrosis through permitting the normal forces excerted during daily movement to promote excessive deformation. Histochemical reaction showed the loss from superficial cartilage of metachromatic staining intensity of proteoglycan integrity in developing osteoarthrosis (Christensen 1980, Colombo 1983A/B, Mankin 1971A, 1981).

A conspicuous change in osteoarthrotic cartilage is in the water content. Normally, the water content of hyaline cartilage is greatest in the surface zones and least in the deep zones (Venn 1977). In human osteoarthrotic cartilage there is an increase in water, a change also found very soon after the experimental induction of joint disease (Mankin 1975, McDevitt 1976). The rate of collagen synthesis is increased in osteoarthrotic human femoral heads in comparison with normal (Mankin 1981). The increased rate is in proportion to the severity of the disease and there is no change in the collagen

phenotype which remains type II, nevertheless, an increased accumulation of type VI collagen has been demonstrated (McDevitt 1988). In experimental dog osteoarthrosis associated with hip dysplasia pro-collagen may accumulate suggesting there is a partial defect in the conversion of pro-collagen to collagen (Miller 1979). The accumulation of this pro-collagen might interfere with normal collagen fibril structure and function, weakening the cartilage and permitting water retention, abnormal swelling and material weakness. The chondrocytes sit in a three-dimensional mesh, requiring a complex inter-dependency between collagens, proteoglycans and water, and clearly any alterations in this normally stable homeostatic environment may lead to osteoarthrosis.

1.8.1.7 Crystals and abnormal materials in osteoarthrosis

In certain situations such as the aggregation of crystals within cartilage there is a clearly demonstrable pathological mechanism for the development of osteoarthrosis. Genetic disorders such as ochronosis precipitate premature cartilage breakdown due to impregnation of cartilage by an oxidised, polmeric, mellalin pigment, derived from accumulated homogentisic acid. Interestingly, in other conditions such as haemosiderosis, haemophilia and haemochromatosis, there is not a similar pattern of disease process. Iron deposition occurs extensively in the synovium but not in the cartilage to any significant degree. Iron is taken up by chondrocytes in haemophilia, for example, but the cells seem to be able to retain metabolic activity in the face of this iron load. Although profound secondary osteoarthrosis does occur, this seems to be due to cartilage breakdown triggered by synovial inflammation and subsequent increased protease activity. Amyloid deposits in articular cartilage of older people has been found to be more common than expected (Goffin 1981) and these aggregates may alter the mechanical properties of cartilage. It is therefore not surprising that amyloid is seen in substantial proportion of knee and hip cartilages examined after arthroplasty for osteoarthrosis (Egen 1982, Ladefoged 1982A/B, 1983). The deposits in knee joint cartilage are often near the cartilage surfaces, and calcium pyrophosphate crystals may lie among amyloid microfibrils. Clearly in these patterns of osteoarthrosis there are demonstrated pathogenic mechanisms.

1.8.1.8 Abnormal geometry

The geometric structure of a synovial joint may be disturbed either because of defected growth, dysplasia or because of injury or destruction. Alterations in joint geometry are likely to promote cartilage breakdown and trauma. This mechanism in the development of osteoarthrosis is clearly demonstrated in animal models of osteoarthrosis with transection of the anterior cruciate ligament in dogs. Likewise in those playing contact sports such as rugby, repetitive ligament injuries lead to joint instability and the development of early osteoarthrosis.

1.8.2 Anatomical changes in osteoarthrosis

Both the primary and secondary patterns of osteoarthrosis lead to molecular cellular and microscopic alterations in the cartilage which manifest themselves as overt, anatomical lesions. The work of Byers' and Meachim gives an account of the development of fibrillation with age. The demonstrable lesions of osteoarthrosis can be said to be the sum of: (1) the area of fibrillation where age alone would not invoke cartilage surface degeneration (2) the lesions exceeding in severity those that occur with age alone, whatever their location (Byers 1970, Meachim 1963, 1972, 1984B). Detailed accounts have been given of the gross anatomical osteoarthrotic changes seen in the major diarthrodial synovial joints (Collins 1949, Gardner 1987, Jaffe 1972, Silberberg 1959, Sokoloff 1969). The majority of these publications have been descriptive and have not considered the underlying molecular processes. Gross pathological diagnosis of osteoarthrosis is best determined by assessing the site and degree of disorder in a particular synovial joint against a baseline structure derived from large cohorts of normal, ageing populations. Such systematic studies have been undertaken on the hip (Byers 1970), the knee (Meachim 1973 1974, 1976), for the ankle (Meachim 1975A) and for the shoulder (Meachim 1973). Collins has made a simple classification of the gross superficial changes of osteoarthrosis through modification of work undertaken by Heine and Fischer (Collins 1949, Heine 1926, Fisher 1929).

Grade one: patches of fibrillation or softening in central areas of articular cartilage, i.e. those not displaying simple ageing changes.

Grade two: fibrillation more pronounced and early marginal chondro-osteophytosis with related synovial cell hyperplasia.

Grade three: changes more severe, commencing exposure of sub-articular bone and more generalised synovial disease with prominent osteophytosis.

Grade four: extensive cartilage loss and bone exposure, eburnation and bone grooving. Associated destruction of intra-articular ligaments, fibrosis or atrophy of synovial tissue and limb abnormalities with shortening and joint sub luxation.

1.8.3 Light microscopic changes in osteoarthrosis

Whatever the joint of anatomical site and irrespective of whether the joint is frequently exposed to gravitational as well as to weight-bearing or non weight-bearing loads, a series of characteristic, inter-related, microscopically recognisable changes follow the alterations in the molecular structure of the joint. These changes which are to be discussed not only affect the hyaline articular cartilage, but also underlying bone, peri-articular structures and the synovium.

1.8.3.1 Chondrocyte proliferation

In both ageing and early in the osteoarthritic process, conspicuous changes in chondrocyte morphology are detectable. In particular in Zone I and II there is initially chondrocyte "drop out" and duplication and triplication of "rests" of chondrocytes. There is clearly an increase in mitotic activity and this has been demonstrated experimentally by the incorporation of radioactive thymidine into rabbits with surgically induced osteoarthrosis (Havdrup 1980). As the osteoarthrotic process progresses there is marked proliferation of chondrocytes particularly in the fibrillary clefts with the formation of well defined microscopic islands of cells with between 5 – 20 cells forming a so-called "chondrocyte clone". Interestingly mitotic figures are absent and tests made with monoclonal antibodies such as Ki 67 that recognises cells in the cycle of division have proved inconclusive. Despite this, it is possible to clearly demonstrate that the affected tissue contained many more cells per unit volume than normal and that there is an increased tissue DNA content which clearly implies cell division (Colombo 1983, Sandy 1984, Silberberg 1964). The stimuli for and mechanisms of, chondrocyte proliferation is poorly understood. A mechanism similar to that by which keratanocytes form clusters without cell division has been considered (Egar 1992, McGuire 1983). This cloning of chondrocytes clearly leads to a profound alteration in the synthesis of macro-molecules such as procollagens and proteoglycans and an understanding of the control mechanisms of this cell proliferation is vital in understanding early phases of osteoarthrosis.

1.8.3.2 Chondrocyte degradation and loss

Studies of chondrocyte mobility in established osteoarthrosis confirm the presence of cell death in Zone I cartilage (Meachin 1984). Matrix disorganisation in zones I and zone II exposes chondrocytes to abnormal environmental stresses and cell loss occurs. Whereas in the deeper zones III and IV cells become swollen and are referred to as hypertrophic chondrocytes. These findings come about from studies on advanced disease in surgical excision specimens from the hip often with vascular or mechanically induced bone necrosis (Meachim 1984). In studies on joints less prone to bone necrosis, there is much less evidence of chondrocyte death (Simon 1976). The true significance of this chondrocyte loss is open to question as the fixation process may produce artefactual "cell death". For example, in formalin-fixed paraffin-embedded, decalcified, dehydrated haematoxylin and eosin stained sections of articular cartilage, conventional light microscopy reveals varying numbers of empty chondrons in the majority of specimens from a subject. However, when formalin-fixed but fully hydrated thin slices of the same cartilage are examined by confocal light microscopic techniques, cell count and cell density is higher (Simon 1976,1982). Certainly cell death occurs in the osteoarthrotic process, however, studies may have exaggerated this due to preparation artefact (Meachim 1984).

1.8.3.3 Cartilage swelling

When there is loss of cross-linking between collagens, there is increased hydration of proteoglycans and consequent swelling of articular cartilage. Some authors recognise this abnormality at the light microscope level by alterations in basophilia of the extracellular matrix (Bennett 1942, Hough 1989) described as "chondromucoid" change. In addition Small "blisters" are said to appear in Zone I due to the tangential orientation of the collagen fibre bundles. Both these phenonoma are subjective and have come under some criticism (Hough 1989).

1.8.3.4 Loss of metachromasia

Studies on ageing tibial condyles show variable loss of metachromasia throughout the matrix whereas with early osteoarthrosis there is a gradual loss of staining from zones I and II downwards (Gardner 1987). As the lesions of osteoarthrosis progress morphologically this reduced matrix staining becomes more conspicuous and there is an inverse correlation between the severity of the histological process and matrix

polysaccharide concentration (Mankin 1971A). Loss of metachromasia and decrease in both safranin O and alcian blue reactivity, are accepted as indices of the progressive leaching out of newly synthesised, but abnormal proteoglycans from their sites within the altered three-dimensional collagen network. (Mankin 1971B, Mallinger 1986, Poole 1970, Getzy 1982, Rosenberg 1971).

1.8.3.5 Fibrillation

Biochemical changes in the matrix are manifested macroscopically as fibrillation. Initially seen as roughening of the cartilage surface on visual inspection. Microscopically one sees the presence of small tangential, oblique and vertical splits in the superficial cartilage matrix. As the disease process progresses, the separation and splitting of the cartilage extends more deeply leaving markedly fibrillated areas of cartilage which contain chondrocyte clones and exhibit a reduction of metachromatic staining (Freeman 1972, Nicholls 1983). Fibrillation advances focally, many cartilage areas are spared, but in affected parts, splitting gradually becomes deeper. The progressive nature of osteoarthrotic fibrillation is thought to distinguish the lesion from the non-progressive lesions of ageing. The presence of fibrillation is often complicated by concurrent disease as amyloid is frequently detected, in the superficial zone I cartilage of elderly subjects, one series of hip joint capsules demonstrating a 28% incidence (Ladefoged 1982A/B, 1983). Advanced fibrillation and cumulative loss of cartilage matrix results in an overall thinning of the cartilage and ultimately leads to full thickness ulceration with exposure of the underlying calcified cartilage. Physiological stresses eventually lead to thickening of this underlying bone leaving a grooved eburnated pattern of subchondral bone.

The rate of progress and pattern of fibrillation is to a degree dependent upon the geometry of the articular surface and an understanding of the shape and direction of this process has been assisted by studies in which a pin has been used to prick the articular surfaces (Hultkranz 1898). When punctured in this way, the elongated split that extends beneath the round, punctured hole is in a direction parallel with the main collagen fibre bundles near the joint surface so that a "split pattern" that is normal for each surface is obtained (Meachim 1974, 1975B, O'Connor 1980 A/B) Deeper extensions of the vertical splits are probably influenced by the collagen fibre pattern and the direction and extent and by the situation of cartilage chrondrons. The chondrons may therefore act as

concentration points for sheer stresses and affects the direction of fibrillation. All fibrillation provides a useful morphological monitor of the phases of osteoarthrosis.

1.8.3.6 Tidemark and subchondral bone

Changes in the tidemark interface occur very early in the osteoarthrotic process. In the pre-fibrillating phase of "early osteoarthrosis", one may see tidemark reduplication and early vascular invasion at the level of the tidemark. As the disease progresses with overt fibrillation there is increasing duplication of the "tidemark" with as many as eight separate well-defined lines seen. In advancing osteoarthrosis, there is increasing disruption of this zone with merging of subchondral bone, calcified and uncalcified cartilage along with islands of new cartilage. Also seen is extensive "invasion" of vascular tissue extending across the tidemark into uncalcified cartilage (Dmitrovsky 1978, Bullough 1983, Broom 1981, Lane 1981, Havelka 1984 and 1986). In parallel with the cartilage splitting are the formation of horizontal splits at the tidemark where the interface of two mechanically different materials, the uncalcified and calcified cartilage lead to a zone of potential weakness. Due to the altered stresses in the underlying subchondral bone, microfracturing occurs, and in response to this there is increased osteoclastic and osteoblastic activity with focal reparative bone remodelling (Saito 1987, Christensen 1980). The increased density of bone beneath osteoarthrotic cartilage may in fact be a local response to the repair of these microfractures and the resultant eburnated bone is composed of an exceptionally dense lamellar matrix with a low cell density. These associated bone changes are inter-related to the progressive cartilage pathology in both severity and degree.

1.8.3.7 Osteophytes and pseudocysts

Osteophytes are bony excrescences that form at the articular margin of most osteoarthrotic joints. It is believed that progressive cartilage loss leads to a redistribution of compressive and sheer forces leading to alterations in mechanical, physical and chemical stresses which promote activation of periosteal osteoprogenitor cells (Fukubayashi 1980). The resultant marginal osteophytes extend and exaggerate the contours of the articulating surfaces in response to abnormal forces and to local pressures. It may be that the cartilaginous islands that form in the chondro-osseous

junction region are also the result of chondro-progenitor cells, promoted by the altered physiological environment.

Irregularly distributed within subarticular bone, often in zones of osteosclerosis, are varying numbers of round or ovoid islands of loose connective tissue which replace bone trabeculae and which have a myxoid appearance, with stellate mesenchymal cells situated in a loose metachromatic connective tissue matrix. Histologically, these structures are "pseudocysts" analogous with those recognised in rheumatoid arthritis. The margins of these pseudocysts may be formed by inert, flattened connective tissue cells, but in addition, osteoclastic bone resorption and osteoblastic new bone formation are often seen (Meachim 1984). The pseudocysts vary in size from 5 – 20 mm and are particularly common in osteoarthrotic femoral heads and encountered to a lesser extent in knees, shoulders and other joints. The exact aetiology of these cystic lesions is unclear, but again, probably represent a bone response to an altered physiological environment with possible bone necrosis and subsequent pseudocyst formation.

1.8.4 Molecular changes in osteoarthrosis

Understanding the molecular changes of osteoarthrosis allows an understanding of the macroscopic and microscopic features of this disease process. Numerous detail reviews exist of alterations in the chemical composition of osteoarthrotic cartilage and its metabolism (Brocklehurst 1984, Hamerman 1989, McKibbin 1979, Muir 1977).

1.8.4.1 Molecular changes – water

Studies have shown that osteoarthrotic cartilage water content is increased and that there is an increased ability to take up water (Mankin 1975, Maroudas 1977, 1987). A correlation has been demonstrated between the degree of osteoarthrosis of femoral condylar cartilage and increasing water content (Brocklehurst 1984). With superficial fibrillation, the water content ranged from 82% in superficial slices and 70% in slices adjacent to the tidemark (These same figures in normal specimens were respectively 78% and 68.5%). With more extensive fibrillation the water content increased to 83% in the superficial cartilage and 75% in deeper cartilage. Furthermore, these studies demonstrated that changes were associated with a decreased rate of glyosaminoglycan

synthesis and content (Brocklehurst 1984). In animal models of osteoarthrosis such as surgically induced canine osteoarthrosis with transection of the cruciate ligament an early increase in water content is noted in the tibial condylar hyaline cartilage prior to microscopic changes in cartilage pathology (Muir 1987). As with ageing alteration in cartilage water content reflects change in the microstructure of the cartilage collagen network allowing greater hydration of proteoglycans.

1.8.4.2 Molecular changes – proteoglycans

As the osteoarthrotic process evolves there are a series of progressive alterations in proteoglycan composition. There is a demonstrable loss of matrix glycosaminoglycans seen by decreased staining by metachromatic dyes such as safranin O (Rosenberg 1971) and alcian blue (Mallinger 1986). This loss of metachromasia is also reflected in a decrease in the fixed charge density (Venn 1977). At a biochemical level there is an increase in chondoitin-four sulphate and a decrease in keratan sulphate representing a change of chondrocyte function as seen by altered cell biosynthesis (Puchtler 1988). Studies undertaken on a dog model of osteoarthrosis showed alterations in the proteoglycan side chains with an initial change in proteoglycan content in central load bearing areas with extension to other anatomical regions (Adams 1987). These fundamental changes in proteoglycan biosynthesis are reflected in altered sulphate incorporation demonstrated by radio-immuno assay techniques (Ryu 1984). Initially, there is an alteration in proteoglycans with smaller chain lengths and a decreased ability to react with hyaluronate. Subsequently there is an increased proteoglycan loss due to degradation mediated by enhanced chrondrocyte enzyme release (Pelletier 1983, 1987). Enzymes involved include acid proteases, cathepsins, proteoglycanases and collagenases. Clearly, these alterations are related with the overall biochemical changes occurring in the evolution of this disease process.

1.8.4.3 Molecular changes – collagen

Early in osteoarthrosis the most pronounced changes that occur in collagen are in the alteration in the three-dimensional structure due to increased protease activity. It appears that there are significant alterations in the proportions of type II, VI, IX and XI collagen (Nemeth-Csoka 1983). In particular is the demonstration in the canine model of osteoarthrosis that there is increased incorporation of H^3-proline type II collagen reflecting an increase rate of synthesis (Chaminade 1982, Muir 1987). This increased

rate of synthesis is greater than the parallel increase in non-collagenous proteins. In addition, similar changes in collagen biosynthesis have also been demonstrated in human osteoarthrotic cartilage with increases being as much as five times normal (Lipiello 1977, Ghadially 1988).

1.9 Tinctorial properties of cartilage

Fixation and decalcification are critical procedures when histological sections are prepared from articular cartilage and ideally, there should be no unrecognised loss of cartilage components or alteration in staining characteristics. Formaldehyde, alcoholic solutions, and quaternary ammonium salt are recommended for the fixation of proteoglycans and glycoproteins (Williams 1956, Allison 1973). Formaldehyde cross-links protein molecules, but carbohydrates do not react directly with formaldehyde (Pearse 1980, Horobin 1982). The protein cross-linking, however, may inhibit the diffusion of some macromolecullar substances not cross-linked by formaldehyde, therefore affecting the staining pattern of proteoglycans (Conklin 1963). Alcohol dissolves water out of proteinaceous material, and denatures them, and carbohydrates are reversibly precipitated, therefore, this is not an ideal agent. Quaternary ammonium salts precipitate acidic oligosaccharides and polysaccharides from binding to the anionic groups of the carbohydrates (Scott 1955). An additional factor is where demineralisation is required as rapid acidic agents cause extensive damage to tissue. However, use of EDTA (ethylenediamine tetra acetic acid) appears to preserve the integrity of the intracellular structures and the stainability of the tissues despite the long demineralisation time required (Charman 1972, Eggert 1981). A variety of combined techniques of fixation and demineralisation are available and in our laboratory the favoured technique is 24-48 hours of isotonic formaldehyde acetic acid fixation, and if demineralisation is required this is then undertaken using EDTA. The theory behind the combination of formaldehyde and acetic acid being that formaldehyde fixation causes cross-linking of amino acid groups of lysine, this inevitably results in shrinkage of the tissue, and this affect is countered by the action of acetic acid which causes a partial unwinding of collagen (Das-Gupta 1990, Kiviranta 1984). Following these fixation and demineralisation processes classic histological staining with haematoxylin and eosin and a number of other histochemical techniques are available for demonstrating both cartilage proteoglycans and collagens (Mallinger 1987, Scott 1972, Christensen 1980,

Jubb 1981, Ippolito 1983). These include picrosirius red, toluidine blue, alcian blue and safranin O.

1.9.1 Picrosirius red

The use of picro-dye reactions for connective tissues rich in collagens has been widely used for many years, recent studies show that the picro-dye, picro-sirius red F3BA is most useful in staining collagens. It has been demonstrated to stain collagens intensely and also has the advantage that it is suitable for polarisation techniques therefore enhancing the collagens (Sweat 1964). Studies on the reactivity of picro-dyes have determined that their molecular configuration and the nature and position of substituents affects their binding capabilities. Physio-chemical studies show that dye-binding is due to non-ionic interactions (van der Waals forces and hydrophobic bonding) Coulomb forces do not impart affinity, in fact, increasing sulphonation actually decreases dye uptake drawing dyes in proximity to non-ionic sites. In addition, bound dyes form aggregates with additional dye ions and these aggregations may vary from two to many powers of ten. This data shows that collagen staining is very much qualitative rather than quantitative (Puchtler 1988). Polarisation techniques have also proved to be more value qualitatively (Dayan 1989).

1.9.2 Toluidine blue

The demonstration of reduced toluidine blue staining of glycosaminoglycans content in osteoarthrotic cartilage is an established, classical histological technique that is used as one criterion for evaluating the severity of the disease. The reproducibility of staining between serial sections from the same tissue specimen remains constant providing sections are at a standard thickness of between 8-10 μ. This dye is able to differentiate fairly subtle differences in metachromasia between pericellular and interterritorial regions of articular cartilage (Getzy 1982). Some studies have shown that there is a semi-quantative relationship between hexuronic acid content of xiphoid cartilage matrix and toluidine blue staining intensity, however, where there is substantial reduction in normal hexuronic acid content, this consistency in staining is lost (Getzy 1982). This may be explained by *in vitro* studies of the interactions of purified mucopolysaccharides with cationic dyes. These studies show a quantitative relationship between the number of dye molecules bound and the number of anionic groups per disaccharide unit; in a system in solution, each dye molecule occupying one negative site. Therefore, the consistency of staining for hexuronic acid is lost with low tissue levels as there must be

no specific binding for acidic groupings such as sulphate groups as the staining intensity is being determined by the presence of free, electro-negative charges of a certain minimum charge density (Poole 1970). This is distinctive from alcian blue that allows staining specificity for chondroitin sulphate and keratan sulphate (Stockwell 1967). Toluidine blue has also been found to be useful in electron microscopic studies allowing a demonstration of electron-dense aggregates of proteoglycans in close proximity to cells which have been stained by toluidine blue (Sheppard 1976A/B). Toluidine blue is a useful dye exhibiting demonstrable and repeatable differences in staining in different regions of the cartilage. It also have the advantage of being sufficiently sensitive to show subtle loss of proteoglycan staining in early osteoarthrosis when there is no superficial cartilage pathology.

1.9.3 Alcian blue

Unlike toluidine blue, alcian blue is a metachromatic stain that allows differentiation of separate cartilage components. Studies using the critical electrolyte concentration technique whereby serial sections are stained with different molar concentrations of magnesium chloride allow for differentiation in staining of chondroitin sulphate and keratan sulphate (Schofield 1975, Whiteman 1973, Scott 1965,1967, 1970, Quintarelli 1964). Using this technique it is possible to demonstrate the altered relationship in content of chondroitin sulphate and keratan sulphate within both different zones and regions of osteoarthrotic and ageing articular cartilage (Mallinger 1986).

1.9.4 Safranin O

Safranin O is the classic dye used routinely for many years as a basic dye for staining glycosaminoglycans (Rosenberg 1971) and assessing their content semiquantitatively. Further studies have shown that the consistency of safranin O staining with serial sections of cartilage from the same affected regions is not as consistently reliable as that using toluidine blue (Getzy 1982). There appears to be some conjecture as to this reliability, some studies suggesting that where there is severe glycosaminoglycan loss there is very little or no safranin O staining suggesting some degree of quantitative assessment is possible (Camplejohn 1988). Microspectrophotometric studies of glycosaminoglycan content also show reliable consistency with minimal non-specific staining (Kiviranta 1985). In addition, like toluidine blue, Safranin O staining may be

used in conjunction with electron-microscopy to show proteoglycan aggregates in the cellular matrix around cells (Sheppard 1976). This histological stain remains a reliable bed rock technique for showing overall broad based changes both in zones and regions of ageing and osteoarthrotic cartilage.

1.10 Knee joint anatomy

The knee is a synovial joint between the femur and the tibia and is the most complex of the human joints. Between the tibial and femoral surfaces are two menisci, which are composed of dense, fibrous tissue. These menisci are C-shaped and are attached to the medial and lateral sides of the articular surfaces and assist in joint articulation and the transfer of mechanical forces between the joint surfaces. Stability to the knee is provided by two internally located cruciate ligaments; the anterior and posterior ligament. Externally to the joint there are ligaments that provide medial and lateral stability. The joint can flex and extend like a hinge and, in addition, the flexed knee can rotate, as in change of direction at speed. In addition to this active rotation, there is passive and inevitable rotation that occurs in straightening the knee in the "screw home" mechanism. During these movements the knee is adapted to be weight-bearing in any position. The plateaux of the tibia possesses two separate articular surfaces, each slightly concave, the medial facet lies wholly on the upper surface of the condyle whereas the lateral facet curves back over the posterior margin of the condyle. The femur has two condyles, separated posteriorly by a deep notch, but fused anteriorly into a trochlear groove for articulation with the patella, the lateral ridge of the trochlear groove is very prominent. The curve of the femoral condyles is cam-shaped when viewed in lateral profile. The distal surface of the medial condyle is narrower, longer and more curve than the lateral condyle – this is for the "screw home" mechanism. The articular surfaces of the patella is divided by a vertical ridge into a large, lateral and a smaller medial surface, this medial surface is divided by a vertical ridge into two smaller areas. The large lateral surface glides around in contact with the lateral condyle of the femur in all ranges of flexion. In extension the area next to it lies on the trochlear, and the most medial of the three surfaces is not in articulation with the femur. In flexion the surface glides into articulation with a medial condyle, and the middle of the three surfaces lies free in the intercondylar notch of the femur. This is an anatomically complex joint and an understanding of the biomechanics of knee

movement is important in appreciating the risk of developing osteoarthrosis in the knee joint (Figures 1-6 and 1-7).

This is reflected in problems which arise either after a significant knee injury with ligament rupture or in competitive sports where ligament laxity occurs such as gymnastics. In both instances there is a markedly increased risk of developing osteoarthrosis earlier in life than would normally be expected. With ligament ruptures numerous surgical procedures are available for ligament reconstruction that may even allow an athlete to compete competitively again – premature osteoarthrosis is likely to follow. These examples demonstrate the importance of maintaining functionally normal anatomy in a complex joint.

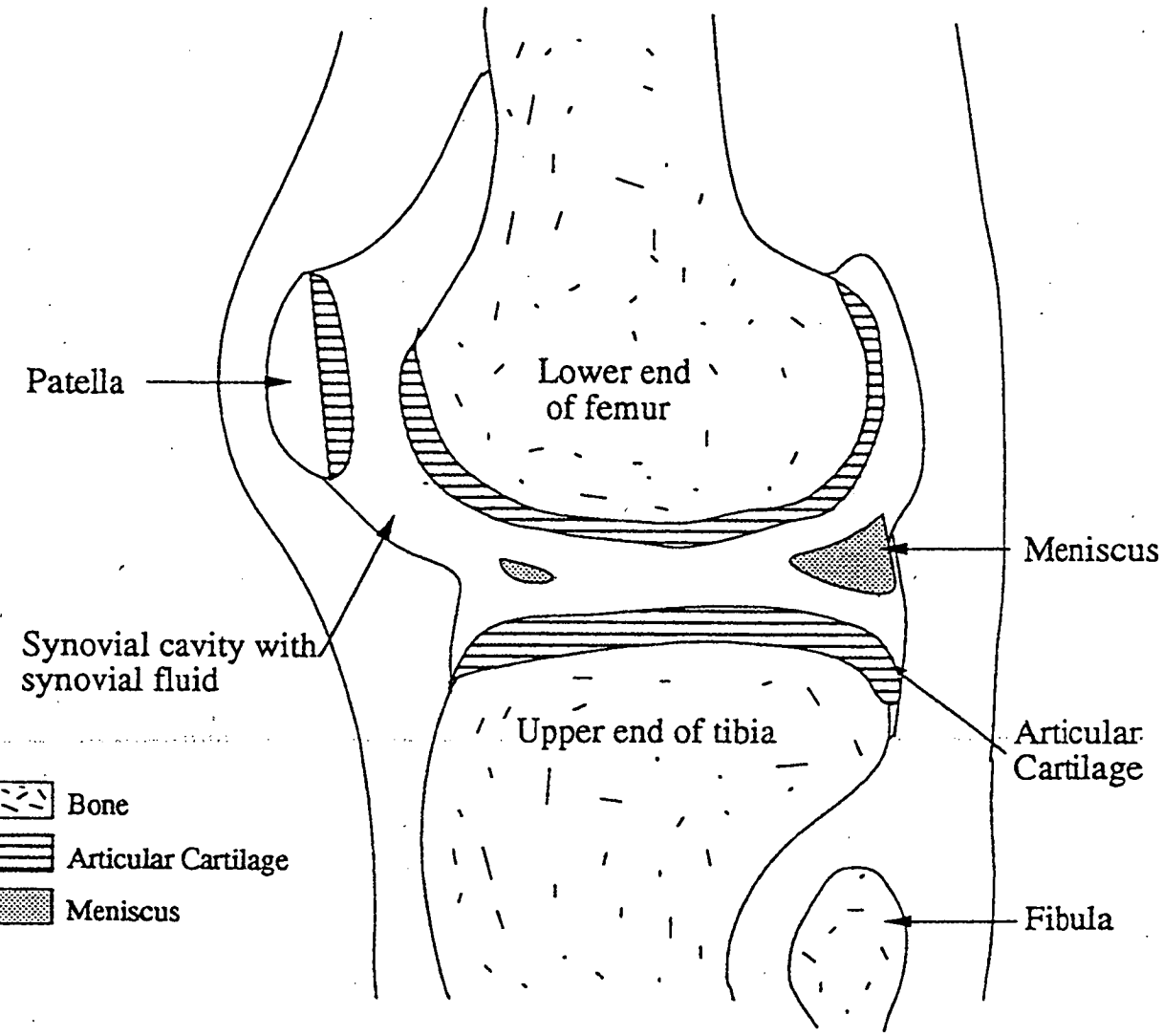
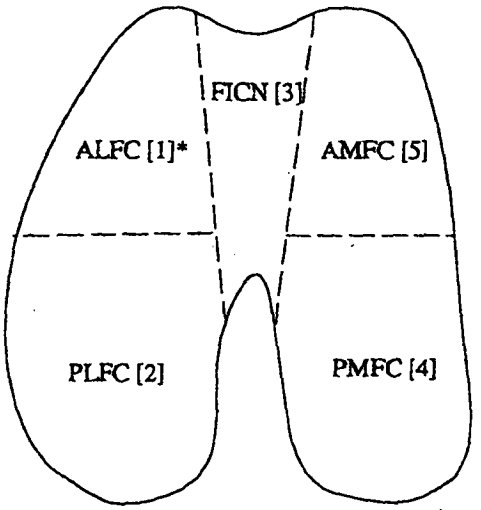


Figure 1-6 Longitudinal Section of Adult Human Knee



FICN - Femoral Inter Condylar Notch

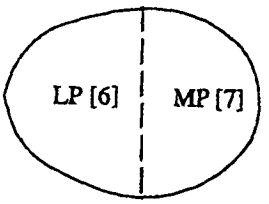
ALFC - Anterior Lateral Femoral Condyle

PLFC - Posterior Lateral Femoral Condyle

AMFC - Anterior Medial Femoral Condyle

PMFC - Posterior Medial Femoral Condyle

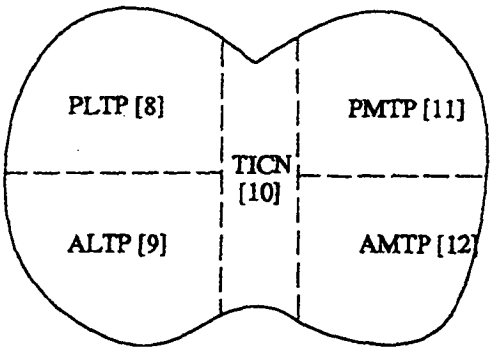
PLAN VIEW OF FEMORAL SURFACE (RIGHT KNEE)



LP - Lateral Patellae

MP - Medial Patellae

PLAN VIEW OF PATELLAR SURFACE (RIGHT KNEE)



TICN - Tibial Inter Condylar Notch

PLTP - Posterior Lateral Tibial Plateaux

ALTP - Anterior Lateral Tibial Plateaux

PMTP - Posterior Medial Tibial Plateaux

AMTP - Anterior Medial Tibial Plateaux

PLAN VIEW OF TIBIAL SURFACE (RIGHT KNEE)

Figure 1-7 Anatomical Surface Regions of the Knee Joint

1.10.1 Biomechanics of the human knee joint

The human knee is a complex articulation composed of two groups of weight-bearing surfaces, the femoro-tibial with lateral and medial components and the patello-femoral. Whereas the loads borne by the femoral head are distributed over a significant part of the main bearing surface, those of the convex femoral condyles are carried by areas that in the normal, erect adult may be no more than 50% of the weight bearing surfaces area of contact. The loading pattern of a normal knee joint is described as a single resultant force, acting through the inter-condylar notch of the knee (Maquet 1984). During a normal two-legged stance the weight supported by the knee joint is approximately 85% of the total body weight (the leg weight below the knees is not included, during normal standing). This force is distributed evenly between the two knees, the resultant force through the knees is also distributed evenly between the two femoral condyles (although this is a simplified approximation of the loading scenario). Stress is distributed along the condylar surfaces with maximum stress at the centre of the condylar surface and diminishing to the periphery and into the inter-condylar areas.

When regarding the knee joint system as a whole, it must be emphasised that in concert with articular cartilage, the meniscus plays a major role in load transference. The contact area of a weight bearing knee in a meniscally deficient joint is approximately 50% of that where the meniscus is intact (Maquet 1984). Meniscally deficient knees therefore generate much higher contract stresses between articular surfaces. Also, structural knee derangement such as meniscal injuries and ligament or tendon injuries can have a detrimental affect on joint movement. In normal walking motion, the instantaneous centres of curvature of the femoral condyle move and describe a cam shaped curve (Dingwell 1994). Where there is a knee injury, motion about an altered centre of rotation can lead to ligament stretching and / or increase compressive loading of the articular surfaces (Franckel 1971). The knee joint undergoes repetitive cyclic loading during normal gait. The period of each cycle has been shown to range of 0.2 seconds (Paul 1965) to 0.5 seconds (Contini 1965). When load is applied to the articular cartilage for such a brief time, although water flow occurs within the tissue, minimal amounts are lost to the synovial cavity. The bi-phasic structural properties can therefore be maintained over prolonged periods of cyclic loading. Articulating joints such as the knee joint can absorb peak loads as high as four times the body weight during motion such as running, or jumping (Maroudas 1979). If cartilage were not present in a joint the result would be a three-fold increase in contact stresses between

the bone surfaces of the joint (Maroudas 1975, 1979). When there is bone to bone contact, such as with severe osteoarthrosis, the stiffness and low radii of curvature of the bone surfaces lead to high contact stresses due to smaller contact areas. Also, since bone is not a compliant, shock absorbing material, such high stresses are likely to lead to mechanical trauma. Articular cartilage deforms under loading, interstitial fluid moves away from the loaded region to an unloaded region. This results in change in the cartilage shape, with an increase in the surface area of contact between the joint surfaces and therefore lowers contact stresses. These biochemical properties of the knee joint are unique in human diarthrodial joints and account for many of the reasons why this is a joint commonly affected by ageing and osteoarthrotic changes.

1.10.2 Knee osteoarthrosis

Osteoarthrosis of the knee is a common and frequently symptomatic illness (Last 1972). Its prevalence increases with age, being virtually negligible in the 25 – 34 age group and increasing to between 20 – 40% in the 55-74 age group. Interestingly, only 34% of people with radiographic changes have symptoms, the percentage being greater the more severe the radiographic changes. The prevalence is higher in men than in women up to the age of approximately 45, after 45, the reverse is true. In addition, after 45, the upswing with age is considered to be more dramatic among women than men. The prevalence of ageing changes and osteoarthrosis has been evaluated by autopsy studies and population based prevalence studies. These studies have assessed the prevalence of cartilaginous change and of osteoarthrosis in persons who have died of other diseases. Because the entire joint can be examined for the local areas of cartilage loss and bony changes, autopsy studies report, on average, much higher estimates of osteoarthrosis prevalence than studies using radiographs. These are purely morphological and fail to consider clinical symptoms in separating ageing and osteoarthoris as separate pathologies (Stankovic 1980, Felson 1988). Original studies found almost universal evidence of cartilaginous damage in those over 65 years of age. Using a more restrictive definition of disease, consisting of cartilaginous erosions with underlying bony changes and osteophytes, other authors found that 60% of males and more than 70% of females had pathological change in the knee consistent with osteoarthrosis, both in the patello-femoral and the tibio-femoral compartments (Felson 1988, Heine 1926, Stankovic 1980). Osteoarthrosis may be divided into primary and secondary disease processes. Certainly in primary knee osteoarthrosis there are clear genetic and familial

dispositions. Secondary osteoarthrosis is very common and is particularly associated with obesity, prior injury particularly to ligaments and menisci, menisectomy, sporting activities and infection. To a lesser extent there is an association with osteochondritis dessicans, developmental abnormalities such as patella malalignment and rarer conditions such as haemophilia.

1.11 Summary

Osteoarthrosis is the most common form of arthritis and is a major cause of morbidity and disability as well as a burden to health. The understanding of the pathogenesis of osteoarthrosis has advanced significantly over the past twenty five years. Although the precise molecular causes of osteoarthrosis remain to be elucidated, it is known that genetic, environmental, metabolic and biochemical factors can contribute to its pathogenesis. An understanding of the normal anatomy, microstructure and biochemistry of articular cartilage is vital in defining the pathology of osteoarthrosis.

Articular cartilage is a unique biological tissue composed of mainly collagen, glycoprotein, proteoglycans, water and other small molecules. Type II collagen is formed into a 3D meshwork by lesser collagen crosslinking and highly hydrated aggregate proteoglycan macromolecules set in the interstices. This unique biochemistry bestows cartilage with its characteristic visco-elastic biomechanical properties. The viability of this matrix is maintained by chondrocytes, the cells of cartilage, which slowly turn the matrix over. Nutrition is said to occur via the synovial surface of the cartilage.

"Hyaline" cartilage sits on the underlying bone being separated by a zone of calcified cartilage. This zone established two clear boundaries, the tidemark (uncalcified-calcified cartilage) and the cement line (calcified-cartilage – bone). These regions have always been considered to be physiologically "inert", however, anatomically they are complex and poorly defined.

There appears to be two distinctive yet interlinking patterns of degeneration that affect articular cartilage, these are ageing and osteoarthrosis. The former sadly is an inevitable result of senescence and is on macroscopic and microscopic grounds is not clearly separated from osteoarthrosis. Ageing of cartilage appears to be a distinctive normal

physiological process and does not inevitably progress to osteoarthrosis. However, the degenerations occurring in the matrix undoubtedly are a risk factor in developing this disease process. Osteoarthrosis appears to be a progressive disease process that ultimately leads to complete loss of cartilage. It may be considered to pass through three stages – early, moderate and severe osteoarthrosis. There are many pathophysiological similarities between ageing and early osteoarthrosis, however, the fine distinctions have not been clearly defined.

The tidemark is a unique region and shows profound change in ageing and osteoarthrosis. Reviews of the literature show the microanatomy of this region is still not fully understood and yet this region undergoes very marked alterations in anatomy in both ageing and early osteoarthrosis.

This chapter has considered in depth the biochemistry and microscopic and macroscopic morphology of cartilage in normality, ageing and osteoarthrosis, an understanding of which is vital in regards to osteoarticular research. The main aims of this thesis are to define the normal anatomy of the tidemark and to undertake a comprehensive review of the histological features in normal, ageing and osteoarthrotic cartilage with particular reference to ageing and early osteoarthrosis. A battery of histochemical staining reactions has been described which allow assessment of the various components of cartilage: matrix, chondrocyte, subchondral bone etc. Evaluation of these stains in a semi-quantitative manner allows the changes in these components to be categorised in the various pathologies. However, these techniques do not allow an assessment of more subtle changes in chondrocytes and matrix cytoskeleton. Carbohydrates are vital components of the proteoglycans of cartilage matrix and the glycoproteins that act as structural molecules in chondrocyte membrane and cytoplasm. An understanding of subtle perturbations in these carbohydrates may increase our understanding of cartilage pathology. Lectins provide a unique investigative tool, as a group they have as a common characteristic the ability to interact specifically and reversibly with carbohydrates. The next chapter provides an overview of lectin histochemistry and considers the use of these sugar binding proteins as a research tool in osteoarticular pathology.

2 CHAPTER TWO: LECTIN HISTOCHEMISTRY

2.1 Introduction to Lectins

Stillmark (1888) originally discovered lectins in the nineteenth century and during the past 100 years they have been known by a variety of names including agglutinins, haemagglutinins, phasins, phytoagglutinins, phytohaemagglutinins, phytotoxins and protectins (Goldstein 1978, Balding 1981). All these titles have drawbacks that make them inappropriate as an umbrella term, and the use is now diminished. Today the most commonly used term is lectin from Latin "to choose, to select", first proposed by Boyd (1954). At the time it was used to describe those plant seed agglutinins that brought about blood-group specific haemagglutination. More recently the term has come to be used more loosely, with no regard to origin or blood-group specificity. A recent attempt at a precise definition of the term lectin (Goldstein 1980) was followed by its adoption with minor amendment (NC-IUB, 1981). The definition accepted is "a lectin is a sugar-binding protein of non-immune origin that agglutinates cells or precipitates glycoconjugates". The definition does not presume a particular function or indicate a particular origin (Franz 1981), and lends itself to experimental confirmation (Dixon 1981). Though widely accepted, the wisdom of defining a lectin by the possession of rather artificial activities has been questioned (Korkourek 1981 & 1983). Thus there remains some dispute as to what is and what is not a lectin, a debate not yet settled.

Lectins are a diverse group of proteins, whose physiological role remains uncertain (Barondes 1981, Graaf 1986, Kolb-Bachofen 1986, Lis 1986). They differ in molecular weight, amino acid composition, carbohydrate content and structure (Goldstein 1978, Lis 1973 & 1986). Thus as a group there are a great many differences, but they have as a common characteristic the ability to interact specifically and reversibly with carbohydrates. Specificity of this interaction was studied at first by using various monosaccharides to inhibit the haemagglutination or glycoconjugate precipitation brought about by a lectin. Using this "hapten" inhibition test, it was evident that the specificity was greater for some parts of a saccharide than others, since a number of similar sugars might bring about inhibition at various

concentrations (Ochoa 1981). Thus lectins were assigned to groups according to a sugar which was the most efficient inhibitor (Goldstein 1978). However, some lectins were found to be poorly inhibited by monosaccharides, and required oligosaccharides to bring about inhibition. As more complex oligosaccharides and glycopeptides came to be used in these studies, it was found that many lectins had a greater affinity for them than for monosaccharides (Kornfeld 1975). This distinction between the relatively low affinity binding of simple sugars and the high affinity binding of more complex glycoconjugates suggested the sugar inhibitor may be greatly different from the actual lectin "receptor". Studies using panels of oligosaccharides or glycopeptides, differing only minimally from one another, have permitted close scrutiny of the sugar sequences important for high affinity binding for various lectins. These studies have revealed the sensitivity of lectins to changes in those saccharide sequences that are part of their binding domain (Gallagher 1984). The classification of lectins utilised in this study is based on their carbohydrate binding specificities and on the most effective monosaccharide inhibitor produced 8 major groups defined on a number of carbohydrate sequences: Group 1 –α–mannose, Group 2 – complex N-linked, Group 3 – N-acetylactosamine, di-N-acetylchitobiose, Group 4 – β-Galactose, Group 5 – N-acetylgalactosamine, Group 6 – fucose, Group 7 – Sialiac acid, Group 8 – α-galactose.

The carbohydrate specificities are based on Landsteiner-type haemagglutination – inhibition assays or polysaccharide precipitations and also on affinity electrophoretic and affinity chromatographic profiles. Most lectins bind to terminal non-reducing sugars although some are capable of binding to internal sequences of glycine. More than one sugar can inhibit an individual lectin but more efficient inhibition can be achieved with complex oligosaccharides or derivative saccharides. Most combine readily with oligosaccharides, e.g. Peanut lectin (AHA) will bind more avidly to Galactosyl β-1, 3 N-galactoseaminyl residues (Galβ 1, 3 Gal NAc α 1-) than to Galactose alone and Erythrina cristagalli (ECA) lectin binds to Galactosyl β-1, 4 N-galactoseaminyl residues (Galβ 1, 4 Gal NAc β-1) more strongly than to Galactoseamine. Hence, lectins, which have the same nominal specificity, may show quite different affinity for the same cells or tissues, because of the more subtle variations capable of being recognised by lectins. Although monosaccharides or

disaccharides may inhibit lectins, the actual binding site of the particular lectin may be quite extended.

Many lectins are considered to have extended binding sites of an essentially cleft-like structure. Consequently, the –OH group at several different positions on oligosaccharide may participate in an interaction with protein and this may impose both requirements for polar substituents at particular positions and configuration constraints. Equally, substituent groups facing away from the cleft-like binding site may play no part in the interaction and may be capable of considerable variation in the nature of the group with which they are being substituted. Most commonly severe constraints are found at C3, C4 and C5 of the sugar, which corresponds with the "competing monosaccharide" and for this reason many lectins, will only interact with these sequences. However, C6 and C2 are also quite commonly restricted to a considerable degree. It is frequently found the anomeric configuration is absolutely defined with regard to naturally occurring glycans but not always with synthetic glycosides. Several examples are available of lectins with an absolute requirement for α-glycosidic linkage to the adjacent sugar but which show the strongest inhibition by the corresponding β-para-nitro-phenyl-glycoside. The reason for this is that the nitro-phenyl group is in a position relative to the distal end of the sugar ring almost exactly the same as that occupied by a polar residue of a sub-terminal sugar. It merely illustrates the requirement for the exact positioning of a number of polar groups along the extended binding site and the relative unimportance of some of the intermediary positions. Because of the limitation of size consequent on the fact that sugars occupy much larger hydrated volumes than amino acids, lectins do not generally show requirements for oligosaccharides much larger than about 7 sugars in length. Even so, their binding sites tend to be larger and hence of higher specificity than most of those found in antibodies directed against glycans.

These properties of lectins have led to varied applications. In biochemistry, the use of lectins has provided a powerful method for the isolation and fractionation of glycoconjugates. Further, the increasingly detailed characterisation of lectin specificity allows their use in the analysis of the carbohydrate component of these molecules (Cummings 1982, Osawa 1987). The selective interaction of certain lectins

with certain cell types provides a basis for cell separation and even fractionation of cell sub-populations (Sharon 1983). This has had important clinical implications (Reisner 1983). Some lectins have proved useful in the identification of micro-organisms, and their use in identification of infectious agents is utilised increasingly. The labelling of lectins has increased their versatility and usefulness (Bernhard 1971, Nicholson 1971). Most work has been carried out using these lectins as a probe of cell membranes. This is particularly so since changes in cell surface glyoconjugates are known to reflect alterations in cell behaviour (Hakomori 1985). Thus lectins have been used to study cell surface changes accompanying development and maturation, and those occurring with functional and pathological states (Coggi 1983, Allison 1986, Damjanov 1986 and 1987). Labelled lectins have also proved useful in the analysis of protein glycosylation within the intracellular compartments (Virtanen 1980, Tartakoff 1983). In comparison with the attention devoted to the cell membrane, using lectin probes, the extracellular matrix has been neglected.

Although a number of studies have been undertaken assessing the staining characteristics of cartilage, none has specifically focused on a broad based study of human articular cartilage in normality and disease. Researchers have looked at human and animal articular growth plate, nasal and tracheal cartilage, tumour cartilage and chondrocyte cultures. All this work has used a limited panel of lectins and has not appraised the various lectin sub-groups and their staining characteristics. This research has focused on 8 groups of lectins with staining affinities for sugar moieties known to occur in osteoarticular tissue. Carbohydrates are known to have a pivotal role in the orientation of proteoglycans in the matrix and other glycoproteins in chondrocytes. Alteration in the stereochemistry of these sugar moieties can be detected by appropriate lectin panels and may further our knowledge of cell-cell and cell-matrix interactions of normal articular cartilage (Yamada 1977, Halberg 1988, Picton 1988, Murata 1984, Mallinger 1986, Schunke 1985).

2.2 APPRAISAL OF SUGAR MOITIES OF GLYCOCONJUGATES

2.2.1 INTRODUCTION

The function of carbohydrate groups on the external plasma membrane of the eukaryotic cells may be to orientate the glucoconjugate such as glycoproteins and glycolipids in membranes and the cell matrix; these groups may also be important for intercellular recognition and so affect tissue development, and could also be important to recognition of self by the immune system.

2.2.2 CARBOHYDRATES

Carbohydrates are classified into three groups:

Monosaccharides divided into:

hexoses – galactose, mannose, glucose;

deoxyhexoses- L-fucose;

hexosamines – N-acetyl glucosamine, N-acetyl galactosamine;

sialic acids – acylneuraminic acid,

pentoses – xylose.

In nature there are over 100 different monosaccharides, however, only about 12 have been found in glycoproteins.

Oligosaccharides more than 8 monosaccharides such as maltose and lactose which can be homologous or heterogenous.

Polysaccharides many linked saccharide residues such as glycogen found in animals and starch in plants.

2.2.3 GLYCOCONJUGATES

Glycoconjugates are carbohydrates which complex with lipids to form glycolipids and to proteins to form glycoproteins. The specialised glycoproteins making up the cartilage matrix are called proteoglycans. These are macromolecular glycoconjugates consisting of specialised polysaccharide chains called glycosaminoglycans attached covalently to a protein core. Proteoglycans also bear covalently attached O- and N-linked oligosaccharides found in all glycoproteins (Gottschalk 1963, Spicer 1992).

2.2.4 GLYCOSAMINOGLYCANS FINE STRUCTURE AND METABOLISM

The structure of glycosaminoglycan polysaccharides have the following biochemical features:

- In principle glycosaminoglycans are repeating disaccharides of a hexuronic acid and a N-acetyl hexosamine sulphate.
- They are highly negatively charged usually having one carboxylate and one sulphate monoester group per repeating disaccharide – accounting for their tinctorial properties.
- Glycosaminoglycans are usually larger than oligosaccharides and never branched (except for keratan) whereas oligosaccharides often are.

Exceptions to these generalisations are:

- Keratan sulphate has galactose residues instead of hexuronate, mainly sulphated at C6 and small but measurable amounts of sialic acid, fucose and mannose forming a branched structure.
- Heparan sulphate and especially heparan carry a number of N-sulphate groups in place of N-acetyl groups, contain iduronic acid in place of glucoronic acid residues with some of the former sulphated.
- Heparin has a higher proportion of N-sulphate and iduronic acid than heparan sulphate. These variations bestow unique oligosaccharide sequences with specific properties.
- Dermatan sulphate has both glucoronic acid and iduronic acid residues.

Therefore glycosaminoglycans consist of repeating sequences forming chondroitin sulphate, dermatan sulphate, heparan sulphate, heparin and keratan sulphate. These molecules possess specialised oligosaccharide sequences that link them to their core proteins via certain amino acids. The amino acids most commonly associated with these linkages with carbohydrates are L-asparagine, L-serine and L-threonine and those which commonly form linkages are 5-hydroxyl-L-lysine and 4-hydroxyl-L-proline (Reid 1990)(Figure 2.1)

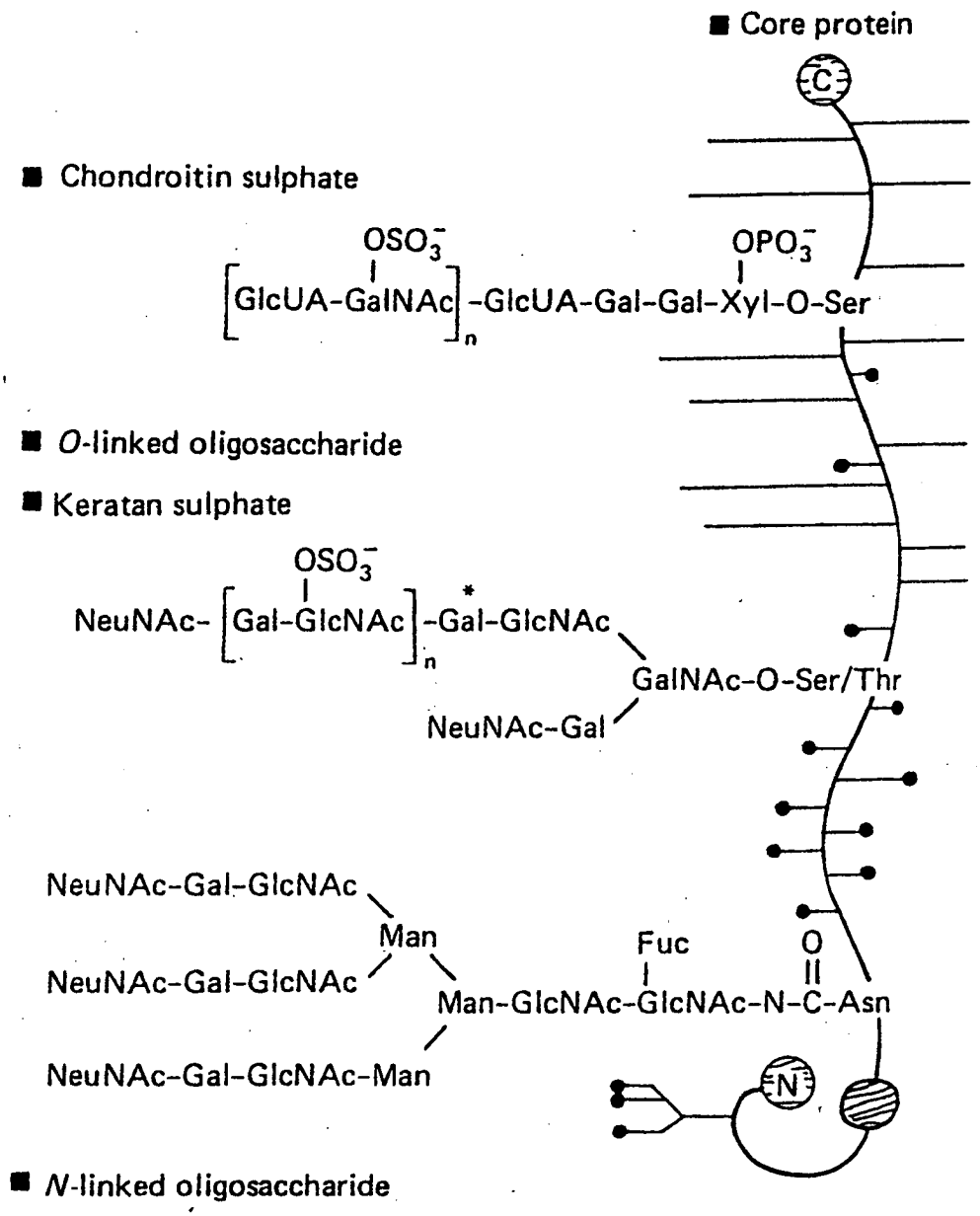


Figure 2-1 Structure of a large aggregating cartilage proteoglycan monomer and saccharide side chains

2.2.5 N- AND O- LINKED OLIGOSACCHARIDES

Studies have shown that there are more N-linked glycoproteins in articular cartilage than O-linked glycoproteins (Picton 1988). O-glycans are most frequently glycoproteins of higher organisms and are glycoprotein secretions (mucins) which are very viscous and have a jelly-like appearance. They are important as lubricants and protect against mechanical and chemical attack. N-glycosidic linkages are more stable than O-glycosidic linkages as demonstrated by the β-elimination technique. Lectins bind to both the N- and O- linked oligosaccharides therefore allow one to assess subtle changes in their chemistry.

There are two types of chemical linkages between the oligosaccharides on glycoproteins via amino acids called N-glycosidic and O-glycosidic. Some molecules contains both N- and O- glycosidic carbohydrate-peptide linkages (e.g. immunoglobulin, erythrocyte membrane glycoproteins). N-acetyl glucosamine usually attaches to asparagine by an N-glycosidic bond and N-acetyl galactosamine usually attaches to serine or threonine by an O-glycosidic bond. N-glycans are glycans, which have an amide group of asparagine, linked to C-1 of N-acetyl-D-glucosamine to form asparagine-N-acetyl glucosamine. By example, this molecule is formed as follows: firstly a lipid carrier (dolichol phosphate) transfers an oligosaccharide unit to the polypeptide chain which has been synthesised on the luminal surface of the endoplasmic reticulum. When the oligosaccharide has attached to the protein, it is further processed as it travels through the endoplasmic reticulum and Golgi apparatus. The oligosaccharide unit carried by the dolichol is composed of 2 N-acetyl glucosamine, 9 mannose and 3 glucose residues which are sequentially added using uridine diphosphate and guanosine diphosphate derived saccharide donors. The final oligosaccharide is then attached to an asparagine residue. In the Golgi apparatus, galactose, fucose and sialic acid are added (Roth 1986). Other monosaccharides can be attached to the asparagine and to the N-acetyl glucosamine residue. N-linked oligosaccharides linked to asparagine all have the same inner-core due to the method of oligosaccharide biosynthesis with the glycoproteins. N-linked glycoproteins have oligosaccharide chains of two types. Firstly, there are high

mannose types with only mannose and N-acetyl glucosamine residues present. Secondly, there are chains that are complex with mannose and N-acetyl glucosamine, sialic acid, galactose and fucose present. An understanding of the exact nature of these chains allows a greater understanding of the interactions of lectins.

2.2.6 CELL-CELL AND CELL-MATRIX INTERACTIONS

Carbohydrate groups appear to be intimately involved in the orientation of molecules involved in cell-cell and cell-matrix interaction. Changes in the carbohydrate group stereochemistry are reflected by alterations in lectin staining patterns and allow an understanding of the evolution of ageing and pathological processes that are occurring in articular cartilage. These changes in carbohydrate chemistry lead to altered cartilage pathology and then in the altered light microscopic appearances of human articular cartilage and its biomechanical properties.

2.3 LECTINS USED IN THIS STUDY

The following is a list of all lectins used in the study. Some were only used in a preliminary trial study before the formal panel of lectins was selected for the main cohorts. The lectins used in this study were chosen from eight groups defined by their sugar binding affinities. These were: Group 1 – mannose, Group 2 – complex N-linked, Group 3 – N-acetyl lactosamine and di-N-acetylchitobiose, Group 4 - β galactose, Group 5 – N-acetyl galactosamine, Group 6 – fucose, Group 7 – sialic acid and Group 8 - α galactose.

Several lectins were chosen from each group dependent on their sugar specificities, lectins within each group often exhibiting subtle distinctions in staining pattern dependent on the orientation of sugar moieties. Not all the lectins in the initial panel stained or two lectins within a group had an identical staining pattern. Following an assessment of each lectin and its staining characteristics a final panel of 19 lectins was chosen from the initial 29.

Acronym	*Source*	
AHA	Arachis hypogaea	Peanut
BSA	Griffonia simplicifolia isolectin - B4/II	
CON A	Canavalia enisiformis	Jack bean
CTA	Erythrina corallendron	Coral tree
DBA	Dolichos biflorus	Horse ground
DSA	Datura stramonium	Jimson weed
ECA	Erythrina cristagalli	Coral tree
GNA	Galanthus nivalis	Snow drop
HAA	Helix aspera	Garden snail
HPA	Helix pomatia	Roman/Edible snail
E/L-PHA	Phaseolus vulgaris (leucoagglutinin)	Kidney bean
LCA	Lens cluinaris	Common lentil
LEA	Lycopersicon esculentum	Tomato
LFA	Limax flavus	Yellow slug
LTA	Tetragonolobus pureus	Lotus

MAA	Maakia amurensis	
MPA	Maclura pomifera	Osage orange
NPA	Narcissus pseudonarcissus	Daffodil
PSA	Pisum sativum	Garden pea
PWM	Phytolacca americana	Pokeweed mitogen
SBA	Glycine max	Soy bean
SNA	Sambucus nigra	Elderberry bark
STA	Solamun tuberosum	Potato
UEA-1	Ulex europaeus – 1	Gorse
VVA B4	Vicia villosa	Hairy vetch
WFA	Wisteria floribunda	Wisteria
WGA	Triticum vulgaris	Wheatgerm agglutinin

2.4 GROUP 1 LECTINS (MANNOSE)

2.4.1 GNA

Snowdrop bulbs (Galanthus nivalis) (Shibuya 1988) accumulate a lectin (GNA) which agglutinates rabbit but not human erythrocytes (Lis 1984, Van Damm 1987). GNA consists of four identical sub units with a molecular weight of 50,000; it is devoid of carbohydrate. GNA precipitates highly branched yeast manans but does not react with most glucans, inhibition experiments showed that D-mannose is an inhibitor of GNA-mannan interactions but that neither N-acetyl-D-mannosamine (NAcMan) nor D-glucose is an inhibitor. Its preference is for Man α 1,3 Man.

2.4.2 NPA

NPA is the lectin derived from the Daffodil (Narcissus pseudonarcissus) (Shibuya 1988). This lectin has a similar range of specificities as GNA, but has preference for the 1,6 as opposed to 1,3 linkage as in Man α 1,6 Man.

2.5 Group 2 Lectins (Complex N-linked)

2.5.1 LCA

This lectin is extracted from the lentil (Lens cluinaris) (Debray 1983, Goldstein 1978, Kornfeld 1981) and consists of two sub-units of total molecular weight of 49,000 daltons. Like Con A (with which it has considerable sequence homology) it is inhibited by α–mannoside which shows distinct variation in its binding sites. It recognises complex N-linked sequences rather than simple high mannose residues detected by Con A. LCA binds to bi- or tri- antennary sequences with terminal Gal residues that may be substituted with neuraminic acid (NeuNAc). Binding of LCA is greatly increased by the presence of α1,6 linked fucose on the N-glycosidically linked (GlcNAc) near the protein core. Whereas Con A may detect by sector sequences, LCA will not.

2.5.2 PSA

This is a lectin derived from the garden pea (Pisum sativum) (Debray 1983, Goldstein 1978, Kornfeld 1981, Trowbridge 1974) and is a tetrameric protein composed of two each of two sub-units (α and β) of total molecular weight 49,000 Daltons. Its binding can be inhibited by α-D-mannosides and in many respects, it is very similar to LCA (with which it shares most of its peptide sequence), in that it binds to bi- and tri-antennary sequences and that α1,6 linked fucose increases its binding. Both lectins recognise the same core structures and the difference in their binding is related to the nature of the linkage of the outer chains. PSA prefers one or two terminal mannose residues but will also bind if there is terminal substitution of the outer chain with either Gal or GalNAc. On the other hand, LCA binding is greater where there is one or two terminal GalNAc residues.

2.5.3 EPHA and LPHA

These are the lectins derived from the red kidney bean (Phaseolus vulgaris) (Yamashita 1983, Cumming 1982, Hammarstrom 1982, Gallagher 1984) and are composed of four isolectins, producing a total molecular weight of approximately 128,000 Daltons. They are glycoproteins, which are mitogenic for lymphocytes, and the isolectins refer to their capacity to agglutinate either leucocytes (L) or erythrocytes (E). Five possible combinations of the isolectins are available (L_4, L_3, E, L_2, E_2, LE_3, E_4) and only the pure form of leucoagglutinating (L_4, LPHA) and erythro-agglutinating (E_4, EPHA) were used in this study. They are unusual in that they are not inhibited by single monosaccharides. They have a complex oligosaccharide binding sites with variations between the two main forms. The minimum binding region required appears to be the disaccharide GlcNAc β 1,2 Man that is present in bi-tri- and tetra-antennary complex N-linked sequences. The leucoagglutinating form (LPHA) does not tolerate bisection of the β-mannose component of the tri-mannosidic sequence attached to the di-N-acetylchitobiose core. There appears to be a requirement for terminal Gal β 1, 3/4 GlcNAc β 1,2 sequences linked to the α mannoses, although terminal sialic acids substitution may also be acceptable. The erythroagluttinating form (EPHA) recognises the disaccharide Gal β 1,4 GlcNAc and also the tri-saccharide Gal β1,4 GlcNAc αDMan. There seems to be a need with

EPHA for bisection of the β-mannose sequence with GlcNAc in β 1,4 linkage and LPHA is more likely to bind to tetra-antennary structures.

2.5.4 Con A

This lectin is derived from the jack bean (Canavalia enisiformis) (Baenziger 1979, Debray 1981, Gallagher 1984, Goldstein 1978, Kornfeld 1975) and was one of the first lectins to be obtained in pure crystalline form. It is one of the most extensively studied lectins in terms of its chemical structure and configuration. It has a molecular weight of approximately 102,000 Daltons and is composed of four polypeptide subunits, each with one carbohydrate-binding site both having Ca^{++} and Mn^{++} ions which are required for binding. This lectin is inhibited by α–glucosyl and α-mannosyl glycosides. Con A recognises a binding site of simple high mannose sequences where it interacts with terminal and two-substituted α-mannosyl residues. It has a preference for short antennae with terminal GlcNAc residues in bi-antennary complex-glycans. Higher degrees of branching or long outer chains prevent binding. It does not appear to be affected by the presence or absence of the N-glycosidic linkage from GlcNAc to the asparadine of the protein backbone, and does not require fucosylation of the N-linked GlcNAc. It is insensitive to bisection of the glycan. Its binding to α–mannosyl terminals may be detected by the use of the exoglycosidase α-mannosidase.

2.6 Group 3 Lectins (N-Acetyl Lactosamine, Di-N Acetylchitobiose)

2.6.1 DSA

DSA is derived from Jimson weed (Datura stramonium) (Debray 1981). DSA, STA and WGA all have similar staining affinities with subtle differences often defined by enzymatic degradations. Binding with increasing affinity (Gal β 1, 4GlcNAc β1)$_n$-/GlcNAc β1,4GlcNAc. This lectin also has some staining affinities with the Group 4 lectins which stain β galactose. This is particularly so for highly branched saccharides with β galactose and reflects that β galactose forms half of N-acetyl lactosamine.

2.6.2 STA

STA is the lectin derived from potato (Solamun tuberosum) (Yamashita 1987). This lectin is similar to WGA with extended binding sites, however, it does seem to have a hydrophobic area that interacts with L-fucose and may cause binding with terminal GlcNAc and NeuNAc in high concentration. It binds to keratan sulphate and therefore sulphation does not greatly affect binding, other glycosaminoglycans do not bind, as they are not 1,4 linked.

2.6.3 WGA

This is the lectin derived from wheatgerm (Triticum vulgaris) (Debray 1981, Bhavanandan 1979, Gallagher 1985, Allen 1973, Goldstein 1978) and it is a pure protein of molecular weight 36,000 Daltons composed of two subunits. The main inhibitory sugar is the di-saccharide di-N-acetylchitobiose $(GlcNAc)_2$. The binding site of WGA is quite complex and it recognises the internal chitobiose structure of N-linked oligosaccharides i.e. GlcNAc β 1-4 GlcNAc, in addition to the repeated sequences of N-acetyl lactosamine and some terminal sialyl residues on the outer chains of both O- and N- linked oligosaccharides. It is likely that the non-reducing terminal N-acetyl neuramanic acid is more important than the internal GlcNAc but also that the relative positions of NeuNAc and GlcNAc are important for the formation of a stable association between the lectin and cells. WGA share some staining affinities with the Group 7 lectins due to its ability to stain sialyl residues. Treatment with neurominidase removes the sialic acid exposing N-acetyl-lactosamine.

2.6.4 LEA

LEA is the lectin derived from tomato (Lycopersicon esculentum) (Ebisu 1978, Murphy 1977). This lectin has a similar pattern of staining to STA.

2.6.5 PWM

This is the lectin derived from Poke weed (Phytolacca americana) (Ebisu 1978, Murphy 1977). The lectin has affinities for (GlcNAc β 1, $4GlcNAc)_1$-3 and (-3Gal β 1,4GlcNAc β 1-)$_n$. It prefers to show strong binding to di-N-Acetylchitobiose cores.

2.6.6 BSA II

(Ebisu 1978, Murphy 1977)

This is one of the lectins derived from the seeds of the Griffonia simplicifolia, previously known as Bandeiraea simplicifolia. There are five possible combinations of two isolectins (A_4, A_3B, A_2, B_2, AB_3, B_4). In the present study both the BSA II and BSA-B_4 isolectin were used in the pilot study. The lectin is a glycoprotein of molecular weight 114,000 Daltons and comprises four units. The main inhibitory sugar is α–DGal and the main binding sites are the terminal α-DGal residues. The B_4 lectin in particular is highly specific for blood group B substances.

2.7 Group 4 Lectins (β Galactose)

2.7.1 MPA

This lectin is derived from the Osage Orange plant (Maclura gomifera) (Grp 5 also) (Sarkar 1981, Young 1989, Mahanta 1990, Bausch 1977) and is composed of two sub units of total molecular weight of 40,000 Daltons. This configuration means that this lectin has interesting properties binding Gal β 1,3 GalNAc α 1- > GalNAc α 1. Therefore there is some homology with the Group 5 lectins and this may lead to paradoxical competitive inhibition as these sub-units are close to each other. Also, saccharides with 1,3 linkage have a perfect match to both sites therefore it is like AHA that binds to Gal β 1,3 GalNAc α 1- stubbs.

2.7.2 ECA

This is a lectin derived from the coral tree (Erythirina cristagalli) (DeBoeck 1984, Iglesias 1982, Kaladas 1982, Ehrlich- Rogozinski 1987) and is a glycoprotein with a molecular weight of approximately 58,000 Daltons composed of two subunits. This lectin does not have a true monosaccharide inhibitor. It appears to have an extended binding site for the di-saccharides Gal β 1,4 GlcNAc β 1-, for which is has a much higher affinity than for the β 1,3 linkage analogue of the di-saccharide. Binding is even greater when there are two or more di-saccharides on the separate outer chains of

oligosaccarides. ECA will tolerate no substitution of β-galactosyl except by a α–galactosyl residue linked at C3.

2.7.3 CTA

CTA is a lectin derived from the coral tree (Erythrina corallendron)(Bhattacharyya 1989). This lectin has a very similar spectrum of specificity to ECA.

2.7.4 AHA

This is a lectin devised from the peanut (Arachis hypogaea) (Lotan 1975, Pereiria 1976) and it is also referred to as peanut agglutinate (PNA). It is a protein of molecular weight 110,000 Daltons and is comprised of four units. The main inhibitory monosaccharide is β D-Gal although the binding site is complementary to the di-saccharides Gal β 1,3 GalNAc α 1-, with much lower affinity for the Gal β 1,3/4 GlcNAc β 1-. The binding site Gal β 1,3 GalNAc is a recognition site for the Thomson-Friedricksen (T antigen) although this is often masked by terminal sialic acid residues thus requiring pre-treatment with neuraminidase for its identification.

2.8 Group 5 Lectins (N-Acetyl Galactosamine)

2.8.1 DBA

This is a lectin derived from the horse gram plant (Dolichos biflorus) (Etzler 1970, Torres 1988, Hammarstrom 1977) and this is capable of agglutinating red blood cells from blood group A individuals (both A_1 and A_2 subgroups). This is a glycoprotein containing 2% (w/w) carbohydrate, and of molecular weight 140,000 Daltons; it is composed of four sub units. It is inhibited by the glycoside of the monosaccharide α-D-GalNAc, with rather less inhibition by α–D-Gal alone. The di-saccharide α 1,4 D-GalNAc β 1,3 Gal also inhibits its binding. Binding is greatly increased where there is α 1-2 fucosyl substitution of the sub terminal galactosyl residue in α 1,3 linkage to the terminal GalNAc. The lectin is capable of recognising both α and β anomers. This lectin shares staining affinities with the Group 6 lectins as the binding site is bifurcated having sites for both GalNAc and fucosyl residues, however, the latter is with lower affinity. It shows some similarity with VVA-B_4 but "sees" larger saccharides with one more sugar.

2.8.2 VVA- B4

This is a lectin of the Hairy Vetch plant (Vicia villosa) (Sueyoshi 1988) and exists in two main forms (A_4, B_4). The A_4 form of the lectin is inhibited by the glycosyde of αD-GalNAc and the main binding site seems to be the terminal non-reducing α D-GalNAc, particularly if there is a sub terminal α 1,3 Gal linkage. This lectin has a high specificity for α–anomers. The B_4 form of lectin recognises the Tn antigen, which is an internal o-glycosidically linked α substituted GalNAc residue. In this study the B_4 form was used.

2.8.3 WFA

WFA is the lectin derived from the Wisteria plant (Wisteria floribunda) (Sugii 1980). WFA lectin is a haemagglutinin and mitogen and has been isolated separately from crude seed extracts by column chromatography and by using immunoadsorbents. The haemagglutinin is a glycoprotein which agglutinates human A, B, and O erythrocytes non-specifically. The lectin has a molecular weight of 68,000 daltons and is made up of two covalently linked subunits of molecular weight 32,000 Daltons each. It has a specificity for GalNAc α l, 6 Gal β 1- that is slightly greater than its specificity for GalNAc α l, 3Gal β 1-.

2.8.4 HPA

HPA is a lectin derived from the Roman snail (Helix pomatia) (Sutton 1992, Torres 1988). Its major specificity is terminal GalNAc α 1-.

2.8.5 SBA

This is a lectin extracted from Soy bean (Glycine max) (Pereira 1974, Sueyoshi 1988) which is a glycoprotein with a molecular weight of 110,000 Daltons, comprised of four sub units. It may be inhibited by the glycoside of either the α or β anomer of D-GalNAc with some preference for the former. The main binding site is for terminal α–D-GalNAc residues but it may also weakly recognise N-Acetyl lactosaminyl and exposed β 1,4 linked galactosyl residues.

2.8.6 HAA

HAA is the lectin derived from the garden snail (Helix aspera) and has virtually the same specificity as HPA. (Sutton 1992, Torres 1988)

2.9 Group 6 Lectins (Fucose)

2.9.1 LTA

This is a lectin extracted from the asparagus or winged pea (lotus plant, Tetragonolobus pureus) (Petryniak 1986). It is a glycoprotein of total molecular weight 295,000 Daltons, composed of three components; a tetrameric protein, a dimeric protein and tetramer. Its main inhibitory sugar is the glycoside methyl α L-fucopyranoside. The predominant binding site is said to be fucose residues in α 1,6 linkage to β GlcNAc in N-glycosidic linkage to asparaginine but it may also bind to fucose residues in α 1,3 linkage to β GlcNAc terminals from outer chains. Whereas UEA tends to recognise single fucosyl residues, LTA prefers more highly fucosylated sequences. It is also specific for Type 2 blood group O (H) where a α 1,2 linked fucose to a terminal Gal with or without a α 1,3 linked fucose to a penultimate GlcNAc residue, is present.

2.9.2 UEA

This is one of the lectins derived from the seeds of the gorse or furze (Ulex europaeus) (Pereira 1978, Petryniak 1986, Born 1987). This is a glycoprotein containing 7.2% (w/w) carbohydrate content and having a molecular weight of about 46,000 Daltons. It may be inhibited by the monosaccharide α L-fucose and has a high affinity for blood group O (H), although it also shows the ability to agglutinate red cells of patients with A_2 and A_2B blood groups. Though it binds to α L- fucosyl residues in 1-linkage to the β GlcNAc residue N-glycosidically linked to asparginine in the protein core, it binds particularly to fucosyl residues attached in 1,2 linkage to β Gal terminals of outer chains (the H - antigen).

2.10 Group 7 Lectins (Sialic acid)

2.10.1 MAA

MAA is derived from the legumenous plant (Maackia amurensis) (Wang 1988). It is one of the few plant lectins to bind sialic acid. It has a slightly different specificity to SNA binding NeuNAc α 2,3 Gal β 1- having a preference for the terminal sialic acid linked (α 2,3) to penultimate D-Galactose residues. Whereas the

preferred site for SNA is to the corresponding (α 2,6) linked oligosaccharides as in the sequence NeuNAc α 2,6 Gal/GalNAC

2.10.2 SNA

SNA is the lectin derived from elderberry bark (Sambucus negra) (Shibuya 1987, Tai 1992). Only a few plant lectins bind to sialic acid. It is not clear whether this is because plants do not contains sialic acids. For many years wheat germ agglutinin (WGA) was the only plant lectin known to have specificity for sialic acids. This specificity is due to the structural similarity of sialic acid to D-GlcNAc which is the preferred ligand for this lectin. SNA is able to bind to the sequence NeuNAc α 2,6 Gal/GalNAc with high specificity. This lectin has a weak affinity for D-Galactose or D-GalNAc and does not bind to either NeuNAc or NeuNG. Another important characteristic of this lectin is that it has a very high affinity for terminal sialic acid linked (α 2,6) to D-Galactose compared to the (α 2,3) linked isomer.

2.10.3 LFA

LFA is the lectin derived from the Yellow Slug (Limax flavus) (Miller 1982, Mandal 1990). The lectin has two equal sized subunits and is highly specific for sialyl residues. Unlike SNA and MAA that are specific to NeuNAc, this lectin will also bind N-glycolyl neuraminic acids.

2.11 Group 8 Lectins (α Galactose)

2.11.1 BSA – B4

This is one of the lectins derived from the seeds of the Griffonia simplicifolia, previosuly known as Bandeiraea simplicifolia. There are five possible combinations of two isolectins (A_4, A_3B, A_2, B_2, AB_3, B_4). In the present study both the BSA II and BSA-B_4 isolectin were used in the pilot study. The lectin is a glycoprotein of molecular weight 114,000 Daltons and comprises four units. The main inhibitory sugar is α DGal and the main binding site is the terminal α DGal residue. The B_4 lectin in particular is highly specific for blood group B substances.

2.12 Methods of Detection of Lectins

Lectin binding to tissue sections may be revealed by a wide variety of methods. The various procedures require different concentrations of lectins and produce different qualities of staining particularly in relation to background levels and permanence of the records. Much of the earlier work was done with direct staining techniques, but more recently indirect methods have been employed successfully.

2.12.1 Direct Staining

2.12.1.1 Fluorescence

In this method used by many of the early investigators, a flourochrome, usually either flourescein or rhodamine was conjugated to the lectin and then applied to the tissue sections. This is a very rapid technique and requires no enzyme blocking, however, there are disadvantages. An expensive flourescence microscope is needed to visualise the results and some of the preparations produced are not permanent. More importantly the concentration of lectin needed is often very high and thus, there is a possibility that low-affinity binding sites are being recognised. Autofluorescence and quenching may cause problems in interpretation.

2.12.1.2 Peroxide reaction

In this technique the enzyme horseradish peroxidase is conjugated to the lectin under investigation and the binding sites are visualised with the chromogen diaminobenzidine in the presence of hydrogen peroxide. The results obtained are permanent, but again high lectin concentrations are necessary which means that the overall system is relatively insensitive and non-specific. Other enzymes may be used e.g. alkaline phosphatase.

2.12.1.3 Gold

This is a very sensitive method of detection of lectin binding sites but is extremely expensive and thus not suitable for general use. Because gold particles are electron dense, this technique is well suited to ultra structural localisation of lectin binding sites. The gold-protein adduct may, however, be quite unstable, causing them to fail or produce high background staining.

2.12.2 Indirect Staining

2.12.2.1 One step antibody technique

An antibody is raised against the lectin required and labelled with one of the following methods described above i.e. flourochrome, peroxidase or gold. This method is more sensitive than direct staining but has the potential difficulty of lack of specificity of the antibody and the need for additional control. Separate antibodies are needed for each lectin thus causing very high cost.

2.12.2.2 Two step antibody technique

With this method, an antibody is raised against the lectin under investigation in one animal species and the second antibody against the immunoglobulin class produced is raised in a second species. The peroxidase-anti-peroxidase (PAP) or alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) enzyme revealing systems is ideally suited to this method. The technique is 10-50 times more sensitive than the direct method but again the possible lack of specificity may be a problem. Even more controls are required and the cost for a study involving several lectins is prohibitive.

2.12.2.3 Avidin – biotin system

The lectin to be investigated is conjugated to biotin while a revealing system such as peroxidase or alkaline phosphatase is conjugated to avidin. These are naturally occurring substances. Avidin is found in egg white and is a glycoprotein of molecular weight 68,000. One molecule of avidin possesses the ability to bind strongly to four molecules of the vitamin biotin thus magnifying the number of binding sites in a complex over the cellular component being visualised. The enzyme labelled avidin is then identified by its reaction with a chromogen. The variation on this method is the use of the avidin-biotin-peroxidase complex system (ABC) which first forms a very large complex over the tissue section before binding to the specific lectin-glycoconjugate site. This large complex may lead to steric hindrance with impairment to the potential binding because of its size. This method allows the use of naturally occurring lectins that have been purified from source. It obviates the need for the production of antibodies that are expensive and potentially non-specific. It also considerably reduces the concentration of lectin required for specific visualisation of binding sites compared with any of the direct methods and thus increases the

sensitivity of the technique. The avidin-biotin-paroxidase method leads to relatively little background staining since endogenous biotin does not occur to any great extent in human articular cartilage by manipulation of the buffering systems, this problem of other techniques is largely bypassed. In the current study, avidin has been used rather than streptavidin. Although avidin has a high isoelectric point (PI) compared with streptavidin and so, tends to bind to polyacids in tissues, the high cost of using streptavidin can be avoided by the use of buffering systems of high ionic strength (Jones 1987A/B, Jeffrey 1987, Leatham 1983A/B).

2.12.3 Controls

The most usual control procedures used in all staining runs are the omission of the biotinyalated lectin and its replacement with buffer. This in effect controls the revealing system and shows the level of background staining which in general terms with the avidin-biotin-peroxide method is very low. This was the control system used in the current research project. A more significant control is use of the specific inhibitory sugar for each particular lectin, although this is not always possible. The lectin in question should bind to its inhibitory sugar, and then when the revealing system is applied, there is an absence or more usually a decrease in the binding of the lectin to the tissue section. This effectively shows that the lectin is binding to its sugar-binding site but does not prove that the sugar in the target glycan is the same either as that added or that to which the lectin is supposed to bind.

2.12.4 Enzymatic Degradations

Various enzymes predominantly exoglycosidases, but in some instances also endoglycosidases, are available for use on tissue sections. Enzymatic degradations cleave specific sugars and where there is already a pattern of staining this may be decreased if the lectins predominate affinity was to the cleaved sugar or increased if additional "concealed" binding sites are revealed. In addition enzymatic degradations may reveal staining where none was previously present. An understanding of each lectin's sugar binding subtleties allows fine distinctions to be made in alteration in staining patterns with lectins in a given group following these enzymatic procedures. In this study the enzymatic degradations which were undertaken were with

neuraminidase, aryl sulfatase, β-elimination, α-fucosidase, α–fucosidase plus neuraminidase and β–galactosidase.

2.12.4.1 Neuraminidase

This is the most commonly used enzyme in this context that acts on terminal non-reducing sialic residues. These are often the final sugar residues incorporated into an oligosaccharide chain and their presence may occlude the binding of the underlying saccharides. Removal of the sialyl residue exposes these "cryptic sequences".

2.12.4.2 Aryl sulphatase

This hydrolase will remove sulphate esters (-O-sulphates) on sugars. It will therefore remove sugars such as keretan sulphate, however, the sulphates of heparinoids are resistant (Schwartz 1974, Toda 1981).

2.12.4.3 β-Elimination

Another possible control is the use of alkaline treatment of the tissues before lectin staining. This is also referred to as the β-elimination reaction and works on the principle that the O- linkage from serine or threonine in the protein backbone to the GalNAc residue in the developing oligosaccharide core is alkaline labile in contrast to the N-linkage from asparagine to GlcNAc which is not. Thus it may be possible to identify those oligosaccharides which are either O- or N- linked to the protein backbone in tissue sections (Downs 1984). Some variations of β-elimination reaction cause minor degradation of some N-glycosides. The method used in the current study has not been shown to cause such degradation but cannot be used quite to completeness in view of unacceptable tissue destruction.

2.12.4.4 α–Fucosidase

The exoglicosidase α-Fucosidase removes a high percentage of α–fucosidase residues from glycoproteins using similar mild acid hydrolysis conditions as for neuraminidase. Therefore it will tend to abolish staining with UEA.

2.12.4.5 α-Fucosidase plus Neuraminidase

The techniques described in 2.11.4.4 and 2.11.4.1 may be undertaken simultaneously instead of undertaking a two step procedure.

2.12.4.6 β-Galactosidase

Exoglicosidases such as β-galactosidase are very useful for cleaving glycosydic linkages of terminal monosaccharides. Most exoglicosidases are specific not only for the monosaccharide but also for the anomeric configuration of the glycosydic linkage. For example, β-galactosidase digests 1,4 links more so than 1,3 links. It does not remove the galactose.

2.13 Lectin Studies in osteoarticular tissues

A number of studies have been undertaken in the osteoarticular field to assess lectin staining characteristics. These have examined human and animal articular cartilage in normality and disease. Assessment has been made of other cartilages including growth plate, tracheal ring, costal, fibro and nasal. Lectins have proved useful in cultured chondrocyte work. These studies have all used relatively small numbers of cases and a limited panel of lectins (Fritz 1988).

2.13.1 Lectin Studies involving hyaline cartilage

A wide variety of studies have been undertaken to study the various components of hyaline cartilage both normal and pathological.

2.13.1.1 Chondrocytes

Chondrocytes from rat and mouse tracheal rings (Yamada 1977 and 1978), rabbit costal (Yamada 1977A), human costal (Mallinger 1986), rat nasal (Mallinger 1986) and human articular cartilages (Schunke 1985) were found to be strongly positive when stained with ConA, although there was considerable variation in intensity of staining. In particular, strongly ConA positive cytoplasmic granules were noted and these were sensitive to digestion with saliva or α- amylase suggesting they must contain a high percentage of glycogen (Yamada 1978A). The plasma membrane, endoplasmic reticulum, golgi apparatus, glycogen and secretory granules in rat tracheal chondrocytes were positive with ConA at the resolution level of the electron microscope (Yamada 1980). Rat and nasal human costal chondrocytes were positive with WGA (Mallinger 1988). Chondrocytes of normal human articular cartilage were negative. Unfibrillated cartilage chondrocytes at the middle and deep zones were positive, however, those of the fibrillated superficial regions remained negative

(Schunke 1985). Few rat tracheal chondrocytes showed any reaction with WGA, only weakly (Ohno 1986). Chondrocytes of normal and fibrillated articular cartilage proved negative both with SBA and AHA (Schunke 1985). In contrast, AHA gave a strong reaction with human costal, and less so with rat nasal, chondrocytes (Mallinger 1986). RCA binding chondrocytes were demonstrated for mouse and rat tracheal and rabbit costal cartilages, with some variation (Yamada 1997), and for human costal and rat nasal cartilages (Mallinger 1986). Chondrocytes, from human costal and rat nasal cartilages, were negative with HPA, whilst UEA 1 produced strongly positive human costal and negative rat nasal chondrocytes (Mallinger 1986).

2.13.1.2 Pericellular matrix

Pericellular matrix is the matrix adjacent to cell and has been described as the inner-layer of the territorial matrix (Mallinger 1986) and the lacunar border (Yamada 1977). It was the most positive of the matrix zones with ConA in rat and mouse trachea, in human costal and rat nasal cartilages (Yamada 1977, Murata 1984, and Mallinger 1986). The pericellular matrix of rat tracheal cartilage was strongly positive (Ohno 1986) and that of human costal and rat nasal cartilages were slightly less so with WGA (Mallinger 1986). RCA showed faint or slightly positive staining for the pericellular matrix of rat and mouse tracheal and rabbit costal cartilages (Yamada 1977) and positive for that of rat nasal and human costal cartilages (Mallinger 1986). SBA and AHA were not found to bind to the matrix of normal human cartilage, but intensely to the superficial matrix of fibrillated cartilage (Schunke 1985, Hoedt-Schmidt 1989). The pericellular matrix of human costal and rat cartilage was positive with AHA (Mallinger 1986). UEA –1 bound to the pericellular matrix of human costal cartilage but gave a negative result with rat nasal cartilage. (Mallinger 1986). Though the extra cellular matrix of normal articular cartilage was negative, the superficial zone of fibrillated cartilage was shown to be strongly positive (Schunke 1985).

2.13.1.3 Territorial matrix

The territorial matrix lies between the pericellular and interterritorial matrices, however, no distinction was made between the territorial and interterritorial matrices in some studies (Yamada 1977, Murata 1984, Mallinger 1986). With ConA this zone was negative in normal articular cartilage and the mid and deep zones of fibrillated

articular cartilage (Schunke 1985). In contrast the territorial matrix of the superficial zone of defibrillated cartilage was strongly positive. WGA bound to the territorial matrix of normal articular cartilage to give an intensely positive reaction, however, in fibrillated articular cartilage while the superficial zone retained this pattern, the mid and deep zones became negative (Schunke 1985). WGA was negative in the territorial matrices of rat nasal and human costal cartilage (Mallinger 1986). Using fluoroscein microscopy the territorial matrix of rat tracheal cartilage exhibited equivocal reactivity with WGA but more abundant reactivity was detected using electron microscopy (Ohno 1986). With RCA-1 the territorial matrix of rat nasal cartilage was negative and that of human costal cartilage was faint (Mallinger 1986). AHA and SBA were negative in normal human articular cartilage, but were intensely positive throughout the matrix of the superficial zone of fibrillating cartilage (Schunke 1985). AHA was negative for the territorial matrices of human costal and rat nasal cartilages (Mallinger 1986). UEA-1 gave a faint reaction to human costal cartilage but was negative in the territorial matrix of rat nasal cartilage (Mallinger 1986).

Studies on human cartilage showed that ConA, LCA, PSA, EPHA, WGA and MPA were similar to each other in their binding pattern (Picton 1988). The superficial matrix showing the greatest amount of lectin binding, however, the binding was often discontinuous and variable in the superficial zones that all lectins use. The staining pattern in this study was noted not to differ significantly with the degree of surface fibrillation. With LPHA the pattern of binding, although present, was less pronounced than the previous group of lectins. A slightly different pattern of staining was seen with VVA-B4, SBA and AHA all of which showed similar binding characteristics with the most pronounced being the superficial matrix and to a much lesser degree the middle and deeper matrix. The DBA, LTA, UEA1 were noted to be negative. Studies have demonstrated that the staining pattern in fixed and processed human articular cartilage was less reactive than in unfixed cartilage (Picton 1988). This may be explained in different ways, fixation and processing may lead to the loss of glycoconjugates which, when present, provide lectin binding sites. Alternatively, and more probably, fixation may cause the lectin binding sites to become less accessible as a consequence of the cross-linking of proteins brought about by formaldehyde (French 1945, Pearse 1980 A & B).

2.13.1.4 Interterritorial matrix

With ConA, normal articular cartilage is positive throughout the interterritorial matrix while in fibrillated articular cartilage this area was negative in mid and deep zones and strongly positive in superficial zones (Schunke 1985). The central interterritorial zone in rat nasal and human costal cartilage were negative but the superficial zones proved faintly positive (Mallinger 1986). In those studies which did not differentiate territorial matrix from interterritorial matrix, the intracellular matrix was shown to be positive with ConA but less so than the pericellular matrix (Yamada 1977 and 1978, Murata 1984). WGA gave a positive reaction with the interterritorial matrix of normal articular cartilage that was less intent than that of the territorial matrix. In the mid and deep zones of fibrillated articular cartilage the reaction with WGA was increased (Schunke 1985). Interterritorial matrix of rat tracheal cartilage was positive with WGA using fluoroscein microscopy and negative using electron microscopy (Ohno 1986). Rat nasal and human costal cartilage was negative (Mallinger 1986). RCA –1 gave a negative reaction with human costal cartilage and a faint reaction with a superficial zone though negative with a central zone, of interterritorial matrix of rat nasal cartilage (Mallinger 1986). The intercellular matrix of mouse and rat tracheal and rabbit costal cartilages were described as faint to positive (Yamada 1977), rat and chick tibial and rabbit costal cartilage was moderate to strongly positive (Murata 1984). No distinction was made between territorial and interterritorial matrices in these latter two studies. SBA and AHA were negative for the extracellular matrix of normal human articular cartilage but were positive in that the superficial zone of fibrillated cartilage (Schunke 1985). Human costal cartilage was negative with AHA in the interterritorial matrix while rat nasal interterritorial matrix was positive in the superficial area only (Mallinger 1986). In the Swarm chondrosarcoma of the rat, SBA discloses "rope like" structures running through the interterritorial matrix. These were absent in the normal rat xyphoid cartilage. UEA binding was absent in the entire extracellular matrix of normal human articular cartilage, but there was greatly increased binding in the superficial zones of fibrillated articular cartilage (Schunke 1985). The interterritorial of rat nasal and human costal cartilages was negative with UEA1 (Mallinger 1986). Studies on human articular cartilage showed that ConA, LCA, PSA, EPHA, WGA, MPA and LPHA staining patterns were similar to that seen

in the territorial matrix but that staining was more pronounced with DBA, LTA and UEA1 (Picton 1988).

2.13.1.5 Fixation Techniques and Enzymatic Degradations

A study undertaken at the University of Manchester was the first work using articular cartilage to critically appraise lectin histochemistry with different fixation techniques and consider the effect of enzymatic degradations. The study focused on the effects of fixation techniques with a relatively small number of cases and limited lectins (Picton 1988), as opposed to the current study which utilised a broad panel of lectins to assess pathological processes in articular cartilage fixed with a standardised procedure.

This study divided the panel of lectins into four groups according to their binding characteristics with cartilage:

Group 1: UEA-1 and LTA were completely negative.

Group 2: ConA, PCA, LCA,EPHA, MPA and WGA showed strong positivity in the pericellular and territorial matrices and in the interterritorial matrix.

Group 3: SBA, AHA, DBA and VVA B4 produced a similar pattern but their binding to the superficial matrix was less evident and

Group 4: LPHA showed strong positive staining of the interterritorial matrix of the middle and deep zones but negative staining of the territorial matrix of the same regions.

Studies on all these groups following the incubation of unfixed articulate cartilage with hyaluronidase and trypsinase showed marked reduction in lectin staining demonstrating reduction in GAG content of the matrix. When the matrix of fixed cartilage was treated with trypsinase the subsequent increase in reactivity towards lectins compared with the matrix of untreated fixed cartilage. This was in stark contrast to the decreased lectin staining of trypsin-digested unfixed articular cartilage and reflects that the trypsin digestion is revealing lectin binding sites in fixed cartilage (Picton 1988).

No change in the lectin binding properties of cartilage was observed after α-mannosidase digestion using ConA, LCA or PSA. This reflects that these lectins were binding to oligosaccharides with structures other than of the high mannose type. No change in the binding pattern of SBA, AHA, DBA, VVA B4 and WGA was noted following neuraminidase digestion. This suggests that N-acetyl neuraminic acid was not a significant binding site for these lectins.

Following β-elimination with the exception of LTA and UEA-1 all the lectins showed moderate staining (ConA, LCA, PSA, EPHA, WGA and MPA slightly more so than SBA, AHA, DBA and VVA B4). Therefore these lectins were binding to oligosaccharides which are linked to protein through alkaline-stable bonds which suggested that the majority of the lectin binding sites were probably to be found on N-linked oligosaccharides.

2.13.2 Lectin studies involving growth plate cartilage chondrocytes

A wide variety of studies have looked at cartilage from growth plates. This cartilage is more metabolically active than hyaline cartilage and generally shows slightly different staining pattern.

2.13.2.1 Chondrocytes

Chondrocytes from swine growth plates were positive throughout the majority of the their cytoplasm with ConA. The nucleus was negative, but the nuclear envelope proved positive. The area of the cytoplasm, which was negative, corresponded with the Golgi apparatus, and with the area that was positive with WGA. Deposits within the cytoplasm, which were positive with ConA, were sensitive to α-amylase, indicative of glycogen (Farnum 1984 and 1985). LCA bound to the rough endoplasmic reticulum, intracellular glycogen and the nuclear envelope in a manner analogous to ConA (Farnhum 1985). A number of lectin were shown to bind to the Golgi apparatus, in addition to WGA, SBA, AHA, RCA1, GSA1, LTA and UEA1 were all found to locate within this compartment. In addition, GSA1, LTA and UEA1 bound to the nucleus (Farnhum 1985)

2.13.2.2 Pericellular matrix

No binding with ConA, LTA or GSA-1 to the pericellular matrix was demonstrated, however, WGA, RCA, AHA and SBA were positive (Farnham 1984 and 1985).

2.13.2.3 Territorial matrix

The territorial matrix was shown to be moderately positive with ConA with exception of the reserve zone. In this area the entire extracellular matrix was negative. Territorial matrix at all levels of the growth plate was moderately positive with WGA (Farnham 1984).

2.13.2.4 Interterritorial matrix

This part of the growth plate matrix corresponds with the longitudinal sector between columns of chondrocytes in zone IV. With ConA the interterritorial and territorial matrices are clearly delineated, the longitudinal sector being intensely positive. WGA did not provide such a difference with the sector often strongly positive (Farnham 1984).

2.13.3 Lectin Studies involving cultured chondrocytes

Chondrocytes from the sternal region of chicken embryos have been used in a study of cartilage matrix synthesis. Lectins were used as a means of identifying intracellular compartments. ConA bound intensely to the peripheral cytoplasm, outside the perinuclear region. This was interpreted as demonstrating the rough endoplasmic reticulum. The other lectins used WGA and RCA, bound to the perinuclear cytoplasm. This was thought to represent the Golgi apparatus (Vertel 1985). Chondrocytes from pig larangyeal cartilage were used to study protein glycan biosynthesis. In this study ricin was used to differentiate between trans- and cis-Golgi apparatus (Ratcliffe 1985). Differences were demonstrated in the lectin binding affinities of the matrix and cells of chondrocytes cultured from the caudal and cephalic end of the sternum (McClure 1993). For example, LPHA bound more intensely to caudal over cephalic chondrocytes whereas GNA bound strongly to intracellular granules in both groups but not to the matrices in either. These studies reflect the different origins of these cell populations.

2.13.4 Lectin Studies involving fibrous cartilage

Mouse semilunar and intraverterbral disc fibrous cartilage have been studied. WGA binding to the interterritorial matrix, with some increase close to cells was noted. Chondrocytes were noted to be positive strongly with ConA (Mallinger 1986)

2.13.5 Lectin studies involving elastic cartilage

Elastic cartilage is found in the ear and nasal septum and a number of studies have been undertaken using rat models.

2.13.5.1 Chondrocytes

Chondrocytes from human elastic cartilage were reactive with ConA and LFA, more faintly with RCA-1 and were negative with AHA, WGA, HPA and UEA-1 (Mallinger 1986). ConA- reactive deposits within the cytoplasm of rat chondrocytes were sensitive to α-amylase (Yamada 1978).

2.13.5.2 Pericellular matrix

Human elastic cartilage in this zone was positive with ConA and strongly positive with RCA –1, AHA, WGA and LFA. Binding of UEA-1 and HPA could not be demonstrated (Mallinger 1986).

2.13.5.3 Territorial matrix

Faint ConA, RCA-1 and LFA reactivity was found but the territorial matrix did not react demonstrably with AHA, UEA-1, WGA or HPA (Malinger 1986).

2.13.5.4 Interterritorial matrix

This part of the matrix proved negative with all the lectins used except WFA, which was faintly reactive (Mallinger 1986). ConA gave a positive reaction with rat elastic cartilage (Stoddart 1973).

2.13.6 Studies in mouse joint cartilage

A number of studies have been undertaken looking at animal models of osteoarthrosis and lectin binding to cartilage (Hoedt-Schmidt 1989A, Schunke 1988). In one study osteoarthrosis was induced in Sprague-Dawley rats by intraarticular injection of sodium iodoacetate. After fourteen weeks the animals were sacrificed and the knee joint cartilage studied against normal controls with a panel of lectins including WGA,

ConA, UEA1, SBA and PNA. WGA matrix staining was strong in normal cartilage but weakly positive in osteoarthrosis suggesting loss of keratan sulphate as WGA binds to the D-N-acetylglucosamine sugar moiety. PNA staining was present only after neuraminidase digestion and matrical staining was strong in osteoarthrotic cartilage. This suggested the galactose residues in keratan sulphate and O-linked oligosaccharides were being revealed. UEA1 was negative or showed minimal staining in normal cartilage but strong matrical staining in osteoarthrosis. This suggested that fucose residues were being revealed on N-linked oligosaccharides. ConA staining was prominent in cells and the matrix in normal specimens and in osteoarthrosis there was increased stain of the superficial zones but decreased staining of deep zones. This suggested an alteration in expression of mannose residues on N-linked oligosaccharides. SBA staining was weak in normal cartilage but was markedly increased in the cells and matrix of the superficial zones. This suggested altered expression of N-acetylgalactosamine particularly on chondroitin sulphate (Hoedt-Schmidt B 1989). Studies on STR/IN mice, which develop spontaneous osteoarthrotic lesions, showed a similar pattern of staining with the same lectin panel (Schunke 1988).

2.14 Summary

This chapter has reviewed the use of lectins in research and appraised their previous use in studies on osteoarticular tissues. Lectins provide a unique research tool which allow studies to be undertaken to assess alteration in tissue carbohydrate chemistry. Macromolecules such as cellular glycoproteins and matrical proteoglycans exhibit very specific staining properties with panels of lectins. As these molecules play a significant role in maintaining the structural orientation of functional molecules (cell receptor, enzymes etc.) alterations in the normal staining pattern of sugars attached to these saccharides side chains could convey information about disease mechanisms. As previous research using lectins has focused more on comparative studies of various cartilages or on the effects of fixation techniques, this study was designed with a different bias. A very broad based panel of lectins was chosen which stained specific sugars found in articular cartilage (Group 1 – mannose, Group 2 – complex N-linked, Group 3 – N-acetyl lactosamine and di-N-acetylchitobiose, Group 4 - β galactose, Group 5 – N-acetyl lactosamine, Group 6 – fucose, Group 7 – sialic acid).

These lectins were utilised to define staining patterns in human knee cartilage collected from a large number of cases relative to other studies. Staining patterns were assessed following formal assessment of cartilage pathology using a panel of classical histochemical stains. It was hoped this focused approach would allow unique observation to be made in demonstrating the difference in ageing and osteoarthrotic cartilage, and in defining the microanatomy of the tidemark. The next chapter discusses the collection of materials and the methods used for histochemical and lectin stains. The parameters that were to be evaluated following these staining technique are then defined.

3 CHAPTER 3: MATERIALS AND METHODS

3.1 INTRODUCTION

There are many aspects of osteoarthrosis that remain poorly understood and as this is such a common disease process causing high morbidity it is an important area of study. The original descriptions of knee joint microanatomy were made in the last century and have been little updated since that time. In particular the tidemark is a poorly understood region, an important boundary between cartilage and bone. Also the subtle changes that occur in early osteoarthrosis and ageing are still poorly described. Both these topics provide a key to understanding the more profound changes that occur in severe osteoarthrosis. In order to research these subjects a review of classical cartilage histology was undertaken using a panel of histochemical stains. Then to further understand the pathological processes a panel of lectins was chosen that stain sugar moieties found in chondrocytes, cartilage and bone. The knee was chosen, as it is a complex joint, which is frequently affected by osteoarthrosis and is easily available for research from surgical and post mortem material.

In studies on human knee joint cartilage considerable variation in physiochemical properties is noted between individuals. The thickness of articular cartilage varies from joint to joint, between weight and non-weight bearing regions, with ageing and in osteoarthrosis. In order to maximise the interpretation of data it is important to collect as broad a number of cases as possible in normal and osteoarthrotic individuals and to sample widely within the joints. The material for this study was collected from surgical knee arthoplasties and post-mortems. The former are now performed for both unicompartmental (UCD) and bicompartmental (BCD) disease therefore material is available from the femoral and tibial components of both compartments. As in UCD overt pathology one compartment antedates the development of overt disease in the other compartment material is available for the study of both "early" and "late" osteoarthrosis. Autopsies in the young and old without joint symptoms provided material for normal controls and assessment of ageing changes.

3.1.1 Research Flow Chart

This research was undertaken in a number of distinct phases indicated in the flow chart below.

Phase 1

Collection of knee joints, clinical assessment and processing of material to paraffin embedding.

↓

Phase 2

Staining with classical histochemical stains and histological assessment into five grades: normal, ageing and early, mild and severe OA. Also S100 staining was performed and 3D reconstruction of the tidemark undertaken.

↓

Phase 3

A preliminary trial of staining with 31 lectins from 8 sub-groups (α mannose, complex N-linked, n-acetyl lactosamine/di N acetylchitobiose, β galactose, n-acetyl galactosamine, fucose, sialic acid and α galactose)

↓

Phase 4

Selection of 19 lectins from these 8 groups, which were used to stain 16, samples each from the 5 histological groups.

↓

Phase 5

Five samples from each of the groups were studied using enzymatic degradations to further understand the staining affinities with the following: galactosidase, fucosidase, aryl sulfatase, β elimination and neurominidase.

3.2 EXPERIMENTAL PREPARATION

3.2.1 SAMPLE ACQUISITION

Knee joints were harvested from 10 hospital autopsy cases [male = 7 (age range 27-70 years, average 50 years), female = 3 (age range 55-67 years, average 62 years)] undertaken at the Manchester Royal Infirmary mortuary by Dr Tim Lyons over a period of five years from 1989 – 1994. A further 37 cases [male = 20 (age range 37-86 years, average 60 years), female = 17 (age range 51-83 years, average 70 years] were obtained from knee joint arthroplasties undertaken by Dr John Fairclough at Cardiff Royal Infirmary over the period of 1989-1994. The Manchester cases were processed immediately and the Cardiff cases were transported in dry ice by courier and were processed the next day.

3.2.2 ANATOMICAL ANALYSIS

Specimens were sectioned on a bandsaw in an AP direction across the centre of each compartment, photographed wet and given an overall macroscopic grade on visual appearance into one of four groups:

- Grade 0 = firm white opalescent intact cartilage
- Grade 1 = soft white slightly translucent intact cartilage
- Grade 2 = very fine superficial fibrillation and softening of cartilage
- Grade 3 = marked surface fibrillation
- Grade 4 = superficial clefts and fissuring
- Grade 5 = deep clefts and fissuring
- Grade 6 = eburnation/ulcers to bone

- Grade 0 = normal
- Grades 1 & 2 = ageing or early OA
- Grades 3 & 4 = mild OA
- Grades 5 & 6 = severe OA

3.2.3 SAMPLE PREPARATION

Full thickness samples of the articular surface, including underlying bone, were taken from 7 distinct areas and ascribed a code.

Anatomical Region	**Code**
Medial tibial plateau	1M
Lateral tibial plateau	1L
Intercondylar notch/patella femoral region	2
Inferior femoral condyle	2M
Inferior lateral femoral condyle	2L
Posterior medial femoral condyle	3M
Lateral femoral condyle	3L

The project number for this Manchester/Cardiff collaborative study was 33 and cases were numbered from 1-47 and anatomical regions coded so they could clearly be identified i.e. project number/case number/ region code = 33/1/1L or 33/42/3L.

3.3 CLASSICAL HISTOLOGY AND HISTOCHEMICAL STAINING

Specimens were fixed in isotonic formaldehyde acetic acid (pH 2.8) and decalcified in Ethelenediamide-Tetra-Acetic Acid (EDTA) for 2-7 days before being paraffin embedded. Using a microtome 10μ mm sections were mounted on glass slides and stained with a panel of classical histochemical stains: Haematoxylin and Eosin (H & E), Toluidine Blue (TB) , Safranin O (SO) and Alcian Blue (AB) were used to stain cartilage matrix glycosoaminoglycans (GAG) and Picrosirius Red (PSR) was used as a collagen stain.

3.3.1 HAEMATOXYLIN AND EOSIN (H & E) STAIN

A standard laboratory method was used – See Appendix A.

3.3.2 TOLUIDINE BLUE (TB) STAIN

A standard laboratory method was used – See Appendix B.

3.3.3 SAFRANIN O (SO) STAIN

A standard laboratory method was used – See Appendix C.

3.3.4 ALCIAN BLUE (TB) STAIN

A standard laboratory method was used – See Appendix D.

3.3.5 PICROSIRIUS RED (PSR) STAIN

A standard laboratory method – See Appendix E.

3.4 S100 STAINING

As there was considerable disruption of normal chondrocyte architecture in osteoarthrosis particularly in the region of the tide mark and as S100 protein is a calcium binding protein, a study was undertaken to assess S100 staining features. This was undertaken on the first 31 cases collected, comprising 12 cases with unilateral disease, 11 cases with bilateral disease and 8 normal joints. From these, the medial and lateral tibial plateaux were stained using standard S100 alkaline phosphatase conjugated avidin staining procedure.

A standard laboratory method was used to stain with S100 – See Appendix F.

3.5 3-D RECONSTRUCTION OF THE TIDEMARK

Interdigitations of uncalcified cartilage followed exactly by the tidemark of normal hyaline articular cartilage were noted to dip into the calcified cartilage. In places these appeared to abut onto subchondral bone and marrow spaces forming a direct communication between the marrow spaces and uncalcified cartilage, a feature which was not well defined. In addition chondrocytes present in these prolongations appeared on two dimensional sections to be entombed in the calcified matrix. In order to understand the anatomy of this region in more detail 3D reconstruction was undertaken, 60 serial sections were taken from block 33/4/1M in a region where these interdigitations were clearly defined. Sections were stained with H & E and captured via a Sony CCD colour video camera mounted onto an Olympus BNZ Microscope.

The solid state CCD camera provides an image measuring 768 x 575 pixels with 8 bits of grey-level information. The digitised image was converted to a mathematical algorithm that allowed alignment to 3D voxels and the construction of a 3D image. The work was undertaken on a unix operated silicon graphics indigo workstation utilising in house software for volumodensimetric studies from the physics department at Manchester University.

3.6 HISTOLOGICAL SCORING PARAMETERS

In order to maximise the data obtained from this study an extremely wide selection of parameters were selected for scoring. Data was scored after assessment of all stains and the scores placed in a worksheet for each case (TABLE 3-1)

Table 3-1 HISTOLOGICAL SCORING CHART

These tables were used for data collection of macroscopic morphology of all specimens studied

NO	GENERAL PARAMETERS					ZP	ZI	ZONE II			ZONE III			ZONE IV			ZV	PG	SPLITS		CHONDROCYTES CLONES					
	GR	SU	MA	CH	CJ	S	MA	PC	TM	IM	PC	TM	IM	PC	TM	IM	MA	MA	HL	VL	FT	IC	II	III	IV	HY
1M																										
IL																										
2																										
3M																										
3L																										
4M																										
4L																										

NO	MATRIX		FIB		CHONDRO-OSSEOUS JUNCTION				CONTACTS			BONE CELLS			SUBCHONDRAL BONE										
	AM	CL	SU	MA	TD	HS	VI	CC	A	B	C	OC	OB	OS	SC BP	NB	WB	EO	NC	MH	MV	FN	CY	FI	MN GC
1M																									
IL																									
2																									
3M																									
3L																									
4M																									
4L																									

ABBREVIATIONS: Anatomical location code (NO); anatomical site; medial and lateral tibial plateaux (1M,1L), intercondylar notch (2), medial and lateral inferior and posterior femoral condylar surfaces (3M, 3L, 4M, 4L); overall grade (GR); surface fibrillation (SU); matrix staining (MA); chondrocytes; chondro-osseous junction (CJ); cartilage zones, zona splendens (ZP), zone I (ZI), zone V (ZV); cartilage regions, pericellular (PC), territorial (TM), interterritorial (IM); cartilage pegs (PG); cartilage splits, horizontal (HL), vertical (VL);chondrocyte clones, frequency (FT), interclonal matrix (IC), zone (II, III, IV), hypertrophic (HY); matrix, acellular (AM), chondrolysis (CL); fibrosis (FIB), surface (SU), matrix (MA); chondro-osseous junction; tidemark (TD), horizontal splits (HS), vascular invasion (VI), chondroclast (CC); bone cells; osteoclast (OC), osteoblast (OB), osteocyte (OS); subchondral bone plate (SCBP); new bone (NB), woven bone (WB), endochrondral ossification (EO), new cartilage (NC), horizontal and vertical microfracture (MH, MV), fat necrosis (FN), cysts (CY), fibrosis (FI), multinucleate giant cells (MNGC).

3.6.1 OVERALL PARAMETERS

A number of scoring parameters were developed to provide a broad based overall assessment of the appearance of articular cartilage. These included an overall grade and assessment of the surface, matrix and cellular components.

3.6.1.1 OVERALL GRADE (GR) OF OSTEOARTHRITIS (OA)

The overall grade of the cartilage was based on an assessment of the surface appearance of the cartilage and changes within the chondrocyte architecture and matrix of the cartilage zones. These were scored from 0-6:

0 = normal

1 = surface intact with mildly disrupted architecture

2 = minor surface abnormalities and mildly disrupted architecture

3 = clefts to transition zone 2 and moderately disrupted architecture

4 = clefts to zone 3 with moderate to severely disrupted architecture

5 = clefts to tidemark and severely disrupted architecture

6 = eburnation

3.6.1.2 SURFACE FIBRILLATION (SU)

Scoring parameters were developed to allow assessment of the changes to the cartilage surface. These were scored from 0-5:

0 = smooth surface

1 = very minor surface defects

2 = clefts to zone 2

3 = clefts to zone 3

4 = clefts to tidemark

5 = eburnation

3.6.1.3 OVERALL MATRIX SCORE (MO)

Scoring parameters were developed to provide overall assessment of the matrical staining pattern. These were scored from 0-4:

0 = strong staining

1 = moderate staining

2 = mild staining

3 = very mild staining

4 = absent staining

3.6.1.4 OVERALL CHONDROCYTE ARCHITECTURE (CH)

Scoring parameters were developed to provide an assessment of the architectural pattern of the chondrocytes. These were scored from 0-4:

0 = normal appearance and zonal distribution

1 = mild loss of architecture and some duplication of chondrocytes

2 = moderate loss of architecture, some loss of columns and small numbers of clones

3 = marked loss of architecture with moderate loss of columns and frequent clones

4 = total disruption of normal architecture

3.6.1.5 CHONDRO-OSSEOUS JUNCTION (CJ)

Scoring parameters were developed for overall assessment of the chondro-osseous junction. These were scored from 0 – 4:

0 = normal anatomy

1 = noticeable changes i.e. tidemark duplication

2 = moderate changes with early vascular invasion and tidemark duplication

3 = changes with marked vascular invasion and tidemark disruption

4 = totally disrupted

3.6.2 MATRIX: CARTILAGE ZONES AND REGIONS

Zona Splendens (ZP); Zone I (ZI); Zone II (ZII); Zone III (ZIII); Zone IV (ZIV); Zone V (ZV); Cartilage Pegs (PG); Pericellular (PC); Territorial (TM); Interterritorial (IM).

The zona splendens is the most superficial component of hyaline cartilage, forming the outer most portion of Zone 1 although frequently defined independently. Hyaline articular cartilage is divided into four zones, (I-IV) and these were sub-divided into regions in relation to the chondrocytes, pericellular is immediately adjacent to the cell, territorial forms the next zone and interterritorial the outer most zone. Cartilage pegs followed by the tidemark dip into the underlying calcified cartilage defined as Zone V. Scores were given for overall matrix staining (MA) in ZP, ZI and ZV and for each region PC, TM and IM in Zones I-IV. Marked changes were seen in matrix staining in developing cartilage pathology and scoring parameters were based on the intensity of staining and were scored from 0-5:

0 = very strong staining

1 = strong staining

2 = moderate staining

3 = mild staining

4 = very mild staining

5 = absent staining

3.6.3 SPLITS IN CARTILAGE (SP)

Vertical (VL) and horizontal splits (HL) within the cartilage were present in diseased joints. These were scored from 0-3:

0 = absent

1 = occasional small

2 = moderate small and occasional medium splits

3 = frequent medium and large splits

3.6.4 CHONDROCYTE CLONES (CC)

The formation of clones of chondrocytes of varying size was a characteristic feature of both ageing and developing osteoarthritis. An assessment was made of the size of the clones within hyaline cartilage zones I-IV. The scoring system was based on the size of the clones. These were scored from 0-4:

0 = absent

1 = occasional small clones

2 = frequent small and occasional medium clones

3 = frequent medium and occasional large clones

4 = frequent large clones

Small = 2-4 cells

Medium = 5-8 cells

Large = greater than 9 cells

3.6.4.1 FREQUENCY OF CLONES (FT)

The frequency of clones was noted. These were scored from 0-4:

0 = absent

1 = infrequent

2 = occasional

3 = frequent

4 = common

This standard graduated scoring system was used for other parameters.

3.6.4.2 INTRACLONAL MATRIX (ZII, ZIII, ZIV)

The matrix within the clones was scored using the same scoring system used for the cartilage matrix (3.7.3.1.)

3.6.5 HYPERTROPHIC CHONDROCYTES (HY)

Large bubbly chondrocytes referred to as hypertrophic chondrocytes were seen in osteoarthrosis. These were scored from 0-4 using the standard graduated scoring system in 3.6.4.1.

3.6.6 ACELLULAR MATRIX (AM)

Areas of acellular matrix were noted within cartilage particularly in well developed osteoarthrosis. These were scored from 0-4:

0 = normal distribution of chondrocytes

1 = noticeable decrease in chondrocytes

2 = moderate decrease in chondrocytes

3 = marked decrease in chondrocytes

4 = complete absence of chondrocytes

3.6.7 CHONDROLYSIS (CL)

Areas of bubbly patchy staining matrix were noted particularly within well established osteoarthrosis. These were scored from 0-3:

0 = absent

1 = mild

2 = moderate

3 = severe

3.6.8 FIBROSIS (FIB) – SURFACE/MATRIX (SF, MF)

Areas of fibrosis were noted both on the surface of the hyaline cartilage and also within the matrix. These were scored from 0 – 3, as for 3.6.7 using a standard graduated system.

0 = absent

1 = mild

2 = moderate

3 = severe

3.6.9 CHONDRO-OSSEOUS JUNCTION (COJ)

The chondro-osseous junction is a complex zone of interdigitation of the uncalcified and calcified cartilages overlaying the subchondral bone. This is characterised by the presence of the tidemark, a clearly defined line separating these two cartilage types. There are changes in this region early in ageing and osteoarthrosis including tidemark duplication, vascular invasion splits and other features each of which are described.

3.6.9.1 TIDEMARK (TD)

A set of scoring parameters was used to assess the degree of replication and disorganisation of the tidemark. These were scored from 0-4:

0 = normal single line

1 = duplication

2 = 2-4 tidemarks

3 = greater than 4 tidemarks

4 = total disorganisation of the tidemark region

3.6.9.2 HORIZONTAL SPLITS (HS)

Horizontal splits were seen at the tidemark. These were scored from 0 – 3:

0 = absent

1 = occasional small

2 = moderate small and medium

3 = frequent small, medium and large

3.6.9.3 VASCULAR INVASION (VI)

Vascular invasion was seen at the tidemark. These areas were scored from 0-4 using the standard graduated scoring system in 3.6.4.1.

3.6.9.4 CHONDROCLASTS (CC)

Multinucleated giant cells referred to as chondroclasts were seen accompanying the small tufts of "invading" vessels. The frequency of these was assessed. The presence or absence of these cells was scored from 0-4 using the standard graduated scoring system described in 3.6.4.1.

3.6.9.5 CONTACTS (A,B,C)

Three types of "contacts" were found to occur between the cartilage and bone:

Type A – complete breaks with vascularised fibrous tissue

Type B – woven bone invested vascular structure

Type C – woven bone plug with fine vascular core

The frequency of these "contacts" was scored from 0-4 using the standard graduated scoring system described in 3.6.4.1.

3.6.10 SUBCHONDRAL BONE PLATE (SCPB)

The subchondral bone plate represents the anatomical region immediately beneath the calcified cartilage. This may become thickened, thinned or absent if there is an associated cyst. These changes were scored from 0-3:

0 = normal

1 = thickened

2 = thinned

3 = absent in places

Considerable disruption of this area occurs in osteoarthrosis and a number of parameters were chosen to assess these changes. The normal cellular component of the bone was reviewed along with pathological features including bone cells, bone changes, microfractures, fat necrosis, cysts, fibrosis and multinucleate giant cells.

3.6.10.1 BONE CELLS [Osteoclasts (OC), osteoblasts (OB), osteocytes (OS)]

The presence of osteoclasts, osteoblasts and osteocytes was recorded in the underlying subchondral bone. The numbers of these cells was scored from 0-3:

0 = normal distribution

1 = mild increase in numbers

2 = moderate increase in numbers

3 = marked increase in numbers

3.6.10.2 NECROTIC AND WOVEN BONE, ENDOCHONDRIAL OSSIFICATION AND NEW CARTILAGE

Within the subchondral bone there were areas of necrotic bone formation (NB), woven bone formation (WB), endochondrial ossification (EO) and new cartilage (NC). These areas were recorded and the size and frequency of foci was scored from 0-3 using a standard graduated scoring system.

0 = absent

1 = small foci

2 = small and medium foci

3 = medium and large foci

3.6.10.3 MICROFRACTURES (HORIZONTAL – MH, VERTICAL – MV)

Both horizontal and vertical fractures were noted in the subchondral bone plate. The frequency of these fractures was scored from 0-3:

0 = absent

1 = occasional small

2 = occasional small and medium

3 = medium and large

3.6.10.4 FAT NECROSIS (FN)

Occasional areas of fat necrosis were seen lying within the medullary cavity. The frequency of these foci was scored from 0-3 using the standard graduated scoring system in 3.6.10.2.

3.6.10.5 CYSTS (CY)

Cyst formation was noted within the subchondral bone plate. The frequency of these cysts was scored from 0-3:

0 = absent

1 = occasional small cysts

2 = frequent small cysts and occasional medium cysts

3 = medium and large sized cysts

3.6.10.6 FIBROSIS (FI)

Areas of fibrosis were noted within marrow spaces. The frequency of these areas was scored from 0-3 using the standard graduated scoring system in 3.6.10.2.

3.6.10.7 MULTINUCLEATED GIANT CELLS (MNGC)

Multinucleate giant cells were noted within subchondral bone plate. The presence or absence of these cells was scored from 0-3:

0 = absent

1 = 1-2 cells

2 = 2-5 cells

3 = 5-10 cells

3.7 LECTINS

Lectins provide a research tool to study cartilage carbohydrate chemistry. Sugar moieties attached to glycoproteins help to orientate functional molecules within the cell cytoplasm, membrane and adjacent matrix. Alteration in the orientation of these saccharides may lead to steric hindrance that can profoundly affect cell-matrix and cell-cell interactions. An understanding of the biochemistry of these glycoproteins in normality and disease may help to understand the disease pathophysiology and to assist in developing treatment regimes.

Classical histochemistry allows a greater understanding of the macroscopic and microscopic changes in ageing and osteoarthrosis, but does not provide information in relation to the sugar chemistry of cartilage. Lectins are an ideal research tool and are used as histochemical markers to demonstrate glycoprotein distribution within the hyaline cartilage and underlying calcified cartilage and subchondral bone. Following histological assessment of the material into five groups, a broad based panel of lectins was chosen from lectins known to have affinities for those sugars commonly found in cartilage. After assessment of this initial group, 19 lectins were chosen so as to obtain the maximum information regarding the cartilage matrix, cells and adjacent structure. Lectins were rejected from the initial panel because they did not stain, or where two lectins had a similar staining pattern only one was chosen. The final panel was considered to be able to provide the maximum information in regard to carbohydrate chemistry.

3.7.1 SELECTION OF MATERIAL FOR LECTIN COHORTS

Overall histological assessment (3.6.1.1) of individual sections allowed sub-division into five groups: normal (group 0); ageing (groups 1 &2); early osteoarthrosis (groups 1 & 2); mild osteoarthrosis (groups 3 & 4) and severe osteoarthrosis (groups 5 & 6). As there had been some differences in the standard of sectioning of the material those cases which provided the most intact and uniform sections were chosen, providing five cohorts of 16 sections each. (TABLE 2-2)

3.7.2 LECTINS USED IN INITIAL TRIAL

An initial trial of 29 lectins was chosen from 8 lectin groups which were known to have affinities for sugars found in cartilage and should therefore provide the greatest

information in osteochondral tissues. For the preliminary trial 2 cases were chosen from each of the 5 groups. A summary of the lectins used in the initial trial is shown, the lectins in bold were used in the final trial.

Group 1- α mannose

- GNA non-reducing terminal α D Man especially the Man 1,3 Man linkage
- *NPA* non-reducing terminal α D Man especially the Man 1,6 Man linkage

Group 2 – complex N-linked

- LCA non-bisected bi/tri antennary N-Glycan (ManGlcNAcGal)
- PSA non-bisected bi/tri antennary N-Glycan (Man)
- EPHA non-bisected tri/tetra antennary N-Glycan
- LPHA bisected bi/tri antennary N-Glycan
- *Con A* α D glucosyl and α D mannosyl residues (some terminal, some 1,2 linked) in high mannose, intermediate and small complex-type sequences.

Group 3 – N-acetyl lactosamine, di-N-acetylchitobiose

- STA Gal β 1,4 GlcNAc β1–/GlcNAc β 1, 4GlcNAc-
- *LEA* Similar to STA.
- DSA (some staining affinity with Group 4) Similar to STA.
- WGA (Some staining affinity with Group 7) N-acetyl neuraminic acid (sialic acid), non-fucosylated di-N-acetylchitobiosyl and N-acetyl lactosamine sequences. GalNAc binds only very weakly. Some sialyl sequences.
- *PWM* (GlcNAc β1,4 GlcNAc)$_1$ –3 and (-3 Gal β1,4 GlcNAc β1-)$_n$ It prefers to show strong binding to di-N-acetylchitobiose cores.
- *BSA II* GlcNAc – (α and β anomers)

Note: STA, LEA, DBA similar but latter two show increasing affinity for multiple branches of repeating NAcLac.

Group 4 - β galactose

- ECA Gal β1,4 GlcNAc
- MPA (some staining affinity with Group 5) Gal β 1,3 GalNAc α1-
- CTA Gal β1,4 GlcNAc, especially in multiple termini

- *AHA* Gal β 1,3 GalNAc α1- > Gal β1,4 GlcNAc β-

Group 5 – N-acetyl-galactosamine

- HPA Terminal GalNAc α 1-
- WFA GalNAc α1,6 Gal β1- > GalNAc α1,3 Gal β1-
- VVA B4 GalNAc α1- and Gal β 1,3 GalNAc α1-
- DBA (some affinity with Group 6) GalNAc α1,3 (LFuc α1,2) Gal β 1,3/4 GalNAc β1- (blood group A-like)
- *HAA* GalNAc- (esp α- anomer), GlcNAc β1-
- *SBA* Terminal GalNAc α1- > Gal α1-

Group 6 – fucose

- LTA Certain fucal.
- *UEA* Fuc α1,2 Gal β 1,4 GlcNAc β1-

Group 7 – sialic acid

- MAA NeuNAc α 2,3 Gal β–
- SNA NeuNAc α 2,6 Gal/GalNAc -
- LFA Certain sialyl terminals

Group 8 - α galactose

- *BSA B4* Gal α 1- (Blood group B – like)

3.7.3 LECTINS USED IN FINAL TRIAL

After assessment of the panel of 29 lectins, 19 were selected from the first 7 groups to collect the most information. The Group 8 lectin did not stain and was not chosen. In some groups more than one lectin was chosen to allow a greater understanding of the geometry and positioning of the saccharides. Enzymatic degradation where sugars are spliced from the glycoprotein provided further information.

Group 1 – GNA

Group 2 – LCA, PSA, EPHA, LPHA

Group 3 – DSA, STA, WGA

Group 4 – MPA, ECA, CTA

Group 5 – DBA, VVAB4, WFA, HPA

Group 6 – LTA

Group 7 – MAA, SNA, LFA

Group 8 – no lectins chosen

3.7.3.1 LECTIN STAINING

A Biotin-Avidin technique was used with a methyl green counter stain. A standard laboratory method – See Appendix G.

3.7.4 LECTINS USED IN ENZYME STUDIES

In order to further define the carbohydrate staining affinities of these lectins a series of enzymatic degradations were performed on selected lectins. A panel of five sections for each of the five cohorts was selected (TABLE 3-3). Each of these degradations described in detail in Chapter Two allowed a greater understanding of the saccharide affinities of the lectin under investigation.

Table 3-2 BLOCKS SELECTED FOR LECTIN COHORTS

Cases selected after histological grading for lectin staining

CODE	NORMAL	AGING	EARLY OA	MOD OA	SEVERE OA
1	2-1M	8-1M	14-1L	6-1M	14-1M
2	4-1L	10-1M	12-1M	6-1L	13-1M
3	11-1L	10-1L	12-1L	8-1L	18-1M
4	15-1M	11-1M	13-1L	32-1M	19-1M
5	15-1L	17-1M	18-1L	34-1M	20-1L
6	16-1M	8-3M	19-1L	35-1M	26-1M
7	16-1L	6-3L	20-1M	32-1L	27-1M
8	17-1L	5-3M	25-1L	34-1L	27-1L
9	10-3M	5-2	46-2	35-1L	22-1M
10	10-3L	10-2	46-1M	33-1M	22-1L
11	4-IC	17-3M	18-2	7-1M	18-3M
12	4-3M	17-4M	18-3L	7-1L	35-3M
13	4-3L	17-3L	29-4L	33-1L	35-2
14	15-4M	17-4L	35-4M	26-1L	44-1M
15	16-4L	11-4M	35-3L	35-4L	44-1L
16	4-MT	8-1M	12-1L	9-1L	6-1M

Table 3-3 BLOCKS SELECTED FOR ENZYME STUDIES

Cases selected for enzyme studies after lectin scoring

CODE	NORMAL	AGING	EARLY OA	MOD OA	SEVERE OA
1	4- 1M	10-1M	20-1M	6-1M	27-1M
2	15-1M	11-1M	25-1L	8-1L	27-1L
3	16-1L	8-3M	46-2	32-1L	44-1M
4	17-1L	17-4M	29-4L	34-1L	44-1L
5	10-3M	17-3L	35-4M	35-1L	13-1M

ABBREVIATIONS: Cohort number (Code); case number (**2 – MT**); medial and lateral tibial plateaux(**1M,1L**); intercondylar notch (**2**), inferior and posterior, medial and lateral femoral condyle (**3M, 3L, 4M, 4L**)

3.7.4.1 GALACTOSIDASE

The lectin selected for galactosidase degradation was MPA.

A standard laboratory method was used – See Appendix H.

3.7.4.2 α-L-FUCOSIDASE

The lectins selected for fucosidase degradation were: ECA , CTA, LTA.
A standard laboratory method was used – See Appendix I.

3.7.4.3 ARYL SULFATASE

The lectin selected for aryl sulfatase degradation was WGA.

A standard laboratory method was used – See Appendix J.

3.7.4.4 β-ELIMINATION

The lectins selected for β-elimination were: ECA, MPA, HPA, VVA B4, DBA, WFA, LTA, SNA, LFA, MAA.

A standard laboratory method was used – See Appendix K.

3.7.4.5 NEURAMINIDASE

The lectins selected for neuraminidase degradation were WGA, ECA, CTA, MPA, HPA, VVA B4, DBA, WFA, SNA, LFA, MAA.

A standard laboratory method was used – See Appendix L.

3.7.4.6 α - L - FUCOSIDASE + NEURAMINIDASE

The lectin selected for this combined degradation was ECA. This method involved degradation with α-L-fucosidase (Appendix I) first, followed by neuraminidase (Appendix L).

3.7.5 LECTIN SCORING

A wide selection of parameters was chosen to provide the maximum information regarding lectin histochemistry. The data was then recorded into worksheets (TABLE 3-4).

Table 3-4 LECTIN SCORING CHARTS

These charts were used to score all parameters in relationship to lectin staining

LEC	SU	ZONE I			ZONE II					ZONE III					ZONE IV					ZONE V	
	S	CY	CM	MA	CY	CM	PC	TM	IM	CY	CM	PC	TM	IM	CY	CM	PC	TM	IM	TD	MA
1																					
2																					
3																					
4																					
5																					
6																					
7																					
8																					
9																					
10																					
11																					
12																					
13																					
14																					
15																					
16																					

LEC	PEGS			SCB		CLONES				CHOSJUNC			NEWCART		
	CY	CM	MA	OS	MA	CY	CM	IC	PC	VE	VM	SP	CY	CM	MA
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16															

ABBREVIATIONS: Lectin cohort code (LEC); surface staining (SU); cartilage zones (I, II, III, IV, V); chondrocytes, cytoplasm (CY), cell membrane (CM); cartilage regions, pericellular (PC), territorial (TM), interterritorial (IM); tidemark (TD); cartilage pegs (PEGS); matrix (MA); subchondral bone (SCB), osteocytes (OS); chondrocyte clones, interclonal matrix (IC), periclonal matrix (PC); chondro-osseous junction (COJ), vessel walls (VE), vessel matrix (VM), tidemark splits (SP); new cartilage (NEWCART)

3.7.5.1 SURFACE (S)

Surface staining intensity was recorded. This was scored from 0-4:

0 = absent

1 = mild

2 = moderate

3 = strong

4 = very strong

The same graduated scoring system was used for other parameters.

3.7.5.2 CHONDROCYTES, CELL MEMBRANE (CM) CELL CYTOPLASM (CY)

The intensity of staining of both CM and CY was recorded and also the percentage of cells staining.

3.7.5.2.1 INTENSITY OF STAINING

Intensity of staining was recorded. This was scored from 0-4 using the standard scoring system in 3.7.5.1.

3.7.5.2.2 PERCENTAGE OF CELLS STAINING

The percentage of cells staining was recorded. This was scored from 0-5:

0 = absent

1 = 1% - 20%

2 = 21% - 40%

3 = 41% - 60%

4 = 61% - 80%

5 = 81% - 100%

In the sheets these are shown as a ratio Intensity/Percentage i.e. 2/3, 3/4 etc.

3.7.5.3 CARTILAGE REGIONS (MA, PC, TM, IM)

The staining intensity of the regions was recorded. These were scored from 0-4 using the standard graduated scoring system in 3.7.5.1.

3.7.5.4 TIDEMARK (TM)

Tide staining intensity was recorded. This was scored from 0-4 using the standard graduated scoring system in 3.7.5.1.

3.7.5.5 SUBCHONDRAL BONE (SCB); OSTEOCYTES (O); MATRIX (MA)

Staining of osteocytes and bone matrix was recorded. This was scored from 0-4 using the standard graduated scoring system in 3.7.5.1.

3.7.5.6 PEGS

The staining of the chondrocyte membrane (CM) and cytoplasm (CY) was recorded as for chondrocytes 3.7.5.2. Matrix was scored as for cartilage regions 3.7.5.3.

3.7.5.7 CLONES

A number of parameters of the clones were recorded.

3.7.5.7.1 CELL MEMBRANE (CM) AND CELL CYTOPLASM (CY)

These were recorded as for chondrocytes 3.7.5.2

3.7.5.7.2 INTRACLONAL MATRIX (IC); PERICELLULAR MATRIX (PC)

The matrix between IC and around PC (chondrocyte clones) was recorded as for cartilage regions 3.7.5.3.

3.7.5.8 CHONDRO-OSSEOUS JUNCTION (COJ)

A number of parameters at the COJ were recorded.

3.7.5.8.1 VASCULAR ENDOTHELIUM (VE)

Staining intensity was recorded. This was scored from 0-4 using the standard graduated scoring system.

3.7.5.8.2 VASCULAR MATRIX (VM)

Staining intensity was recorded. This was scored from 0-4 using the standard graduated scoring system.

3.7.5.8.3 SPLITS (SP)

The frequency and size of splits was recorded. This was scored from 0-4:

0 = absent

1 = small, infrequent

2 = small and medium, more frequent

3 = small, medium and large, frequent

4 = medium and large, very frequent

3.7.5.9 NEW CARTILAGE (NC)

Parameters of NC for both chondrocytes and matrix were recorded as for 3.7.5.2 and 3.7.5.3.

3.7.5.10 MATERIALS AND SUPPLIERS

The main materials and suppliers of equipment in this study are recorded in Appendix M.

3.8 SUMMARY

This chapter has documented the source of the material and the experimental procedures. After material had been collected from post mortems or arthroplasties it was assessed macroscopically before being processed to paraffin blocks. An initial histological assessment was undertaken using H & E, glycosaminoglycan and collagen stains that allowed division of the material into five groups: normal, ageing and early, moderate and severe osteoarthrosis. A very broad panel of parameters was used in order to assess the tidemark region and document features of ageing and early osteoarthrosis.

As S100 protein stains chondrocytes and as the normal cellular architecture was disrupted in osteoarthrosis, a number of cases were studied from the normal, early and severe OA groups. The microanatomy of the tidemark was poorly defined, in particular, the association between the uncalcified chondrocyte pegs that dip into calcified cartilage and the marrow spaces was unclear. The normal anatomy of this region was studied and a 3D reconstruction of this area was undertaken to define the association.

In order to then evaluate the carbohydrate chemistry of cartilage in normal, ageing and disease, lectin histochemistry was undertaken. From a broad panel of 29 lectins

19 were chosen from 7 groups (characterised by their affinities for certain sugars) which were best considered to characterise the carbohydrates found in cartilage. Then a number of enzymatic degradations were undertaken to further differentiate staining characteristics within individual lectin subgroups.

All the data was recorded onto worksheets and the results are documented in Chapter 4. The material is presented in a semiquantitative descriptive format with a summary of findings, tables and illustrations.

4 CHAPTER 4: RESULTS

4.1 INTRODUCTION

The previous chapters have reviewed; one, human cartilage in normality and disease; two, lectins and their use in osteoarticular research; and three, materials and methods used in the collection, staining and evaluation of specimens of human knee joints obtained at autopsy and arthroplasty. This chapter documents the results of the various sections of the study: morphological assessment, S100 protein staining, normal anatomy and 3D structure of the tidemark and lectin histochemistry. In each section there is a discussion of the results followed by a summary of the key findings in a table and photographs of key features.

4.2 MACROSCOPIC MORPHOLOGY

After collection of specimens either from autopsies undertaken in Manchester or arthroplasties performed in Cardiff, material was photographed and given an overall macroscopic grade on visual appearance (TABLE 4-1). This macroscopic assessment allowed classification into 6 grades, which could then be divided into 5 clinical groups: normals, ageing (asymptomatic post mortem specimens), early osteoarthrosis, moderate osteoarthrosis and severe osteoarthrosis. In each joint compartment there were three anatomical areas assessed – the area most severely affected was chosen to give the overall compartment grade recorded in Table 4-2.

As a total of 47 joints were studied with up to 7 blocks being taken per joint (anatomical area 1M, 1L, 2M, 2L, 3M, 3L and 2) a good selection of blocks were obtained for each disease parameter studied. For example, where moderate or severe changes were seen on opposing weight bearing surfaces the posterior non-weight bearing surface of the femur only showed early change. (The significance of these findings will be considered in more detail in the discussion). The joint compartments were scored as follows: normals 6 cases (blocks 28, average age 41), ageing 5 cases (blocks 17, average age 64), early OA 15 cases (blocks 69, average age 52), moderate OA 30 cases (blocks 94, average age 65) and severe OA 33 cases (blocks 72, average age 63).

Macroscopic normals in this series where characterised by the joint surface being covered in opalescent, smooth, firm, white cartilage. The ageing group was characterised by slight yellowing of the articular cartilage and surface disruption with early fibrillation and slight softening of the articular surface. In the early OA group, in comparison to the ageing group, the surface appeared to be whiter and more translucent, slightly less fibrillated and softer to gentle indentation. The moderate and severe groups were characterised by the presence of pits and erosions as well as fibrillation, perpendicular fissuring and yellowing of the cartilage. Progression of these changes was characterised by gradual extension of the fissures ultimately through the cartilage to the subchondral bone. Initially there was distinctive superficial fibrillation and thinning of the cartilage and as the process evolved the fibrillation became more pronounced the erosions coalesced and the cartilage thinning was more profound with fragmentation of superficial tissue. Eventually in severe disease underlying bone was revealed and this took on the classical "ivory-like" appearance of eburnated bone. (Figures 4-1 to 4-8 show plain and side views of normal and osteoarthrotic cartilage).

The most distinctive feature was the difference in appearance between the ageing and early OA groups. In the former the cartilage was clearly firmer to gentle pressure and showed distinctive early fibrillation. Whereas in the latter the cartilage remained white and relatively smooth but was distinctly softer on gentle pressure with a slower viscoelastic response after gentle indentations were made with a metal probe (personal intra-operative experience).

Table 4-1 Source of Specimens and Clinical Grade

CASE NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SOURCE	C	C	C	MA	MA	C	C	MA	C	MA	MA	C	C	MA	MA	MA
SEX	F	M	F	M	F	M	F	M	M	M	M	M	M	F	M	M
AGE	73	47	75	27	67	70	51	62	55	67	65	72	64	55	35	35
CLINICAL	B	U	B	N	N	B	U	N/A	U	N/A	N/A	U	U	B	N	N

CASE NO.	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
SOURCE	MA	C	C	C	C	MA	C	C	C	C	C	C	C	C	C	C
SEX	M	M	F	M	F	F	M	M	M	M	F	F	M	F	F	M
AGE	70	52	78	68	78	65	42	24	52	68	57	80	69	57	67	86
CLINICAL	N/A	U	U	U	B	B	U	U	U	B	B	B	B	B	B	B

CASE NO.	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
SOURCE	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
SEX	M	M	M	F	F	F	M	M	F	F	F	F	F	M	M
AGE	70	65	62	76	75	69	85	69	54	83	83	75	73	47	37
CLINICAL	B	B	B	B	B	B	B	B	B	B	B	B	B	U	B

Abbreviations: Cardiff arthroplasties (C), Manchester autopsies (MA), Male (M), Female (F), Bilateral OA (B), Unilateral OA (U), Normals (N), Ageing (A)

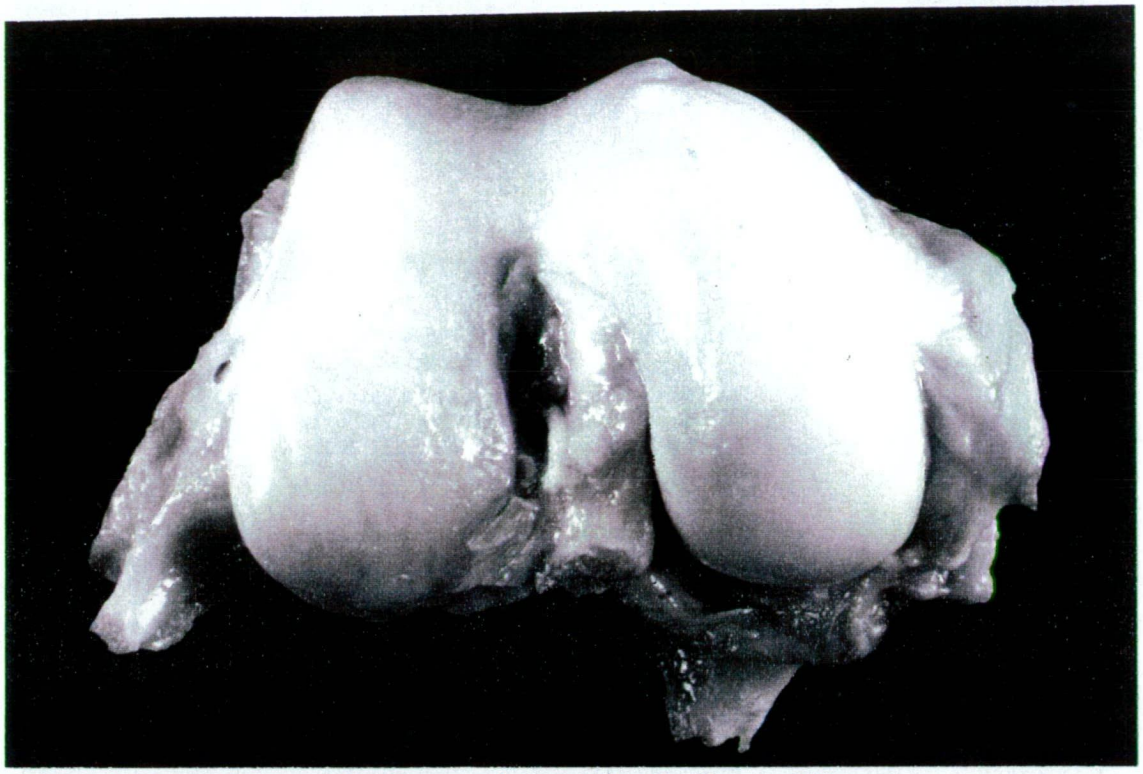


Figure 4-1 Plan View of the Femoral Condyle of a Normal Knee

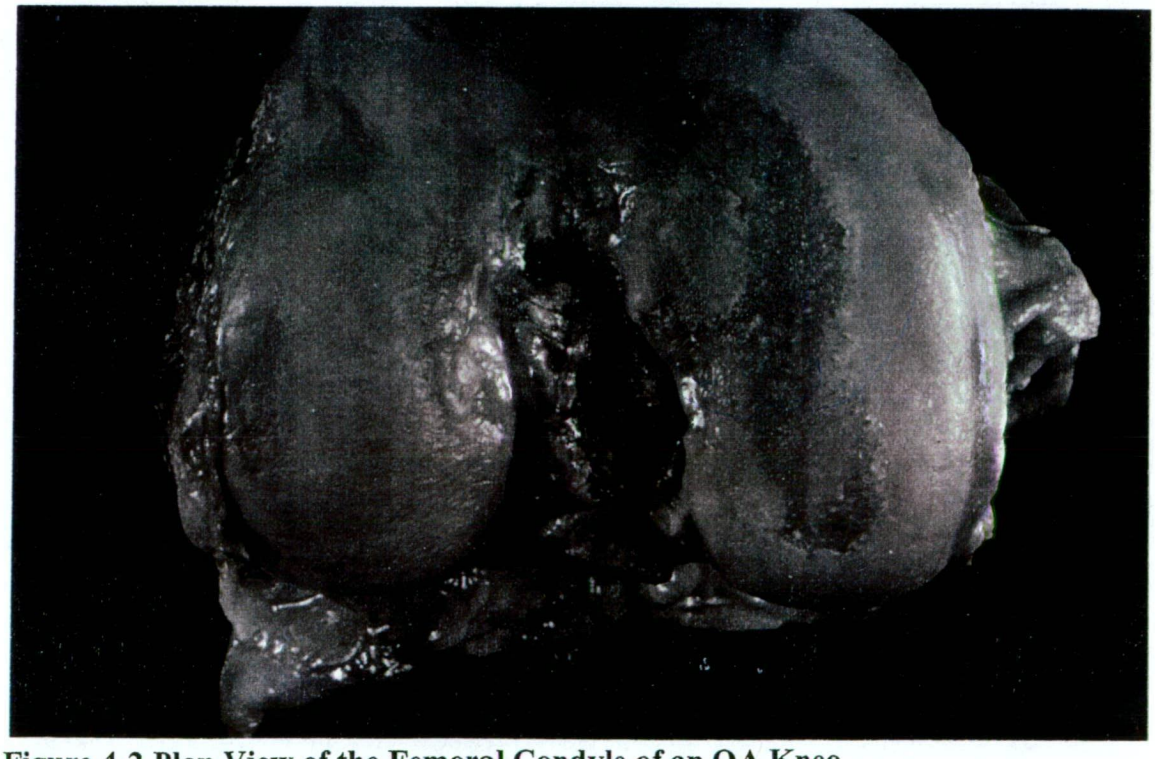


Figure 4-2 Plan View of the Femoral Condyle of an OA Knee

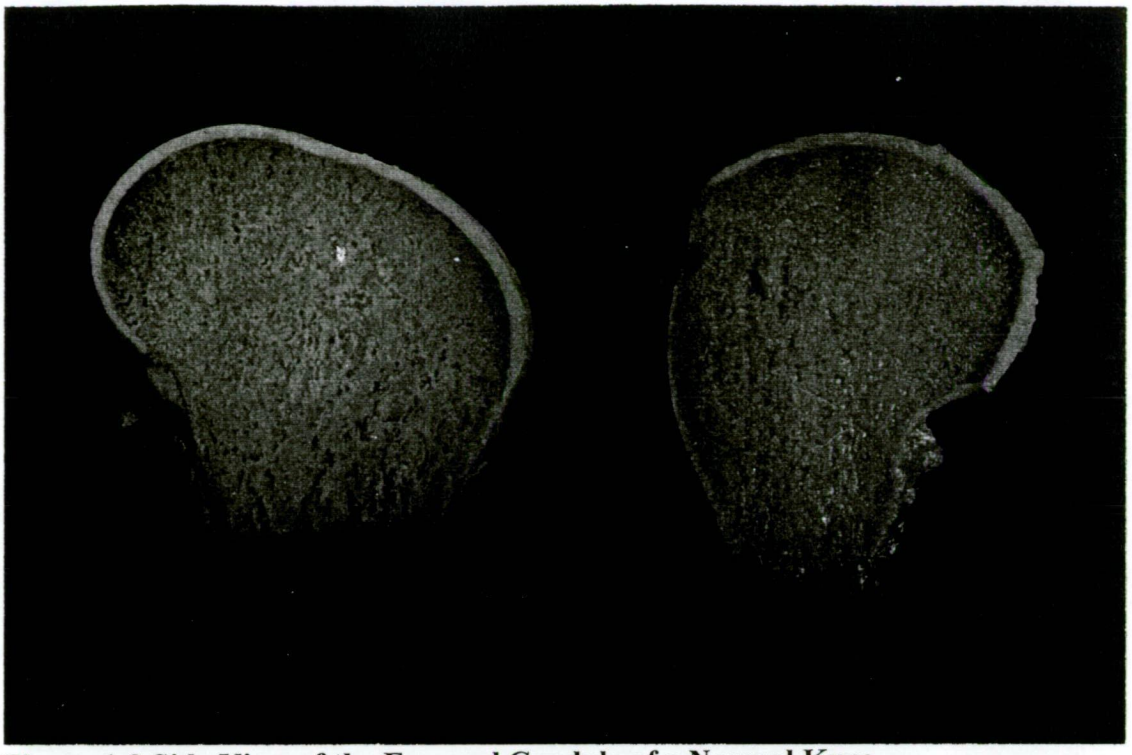


Figure 4-3 Side View of the Femoral Condyle of a Normal Knee

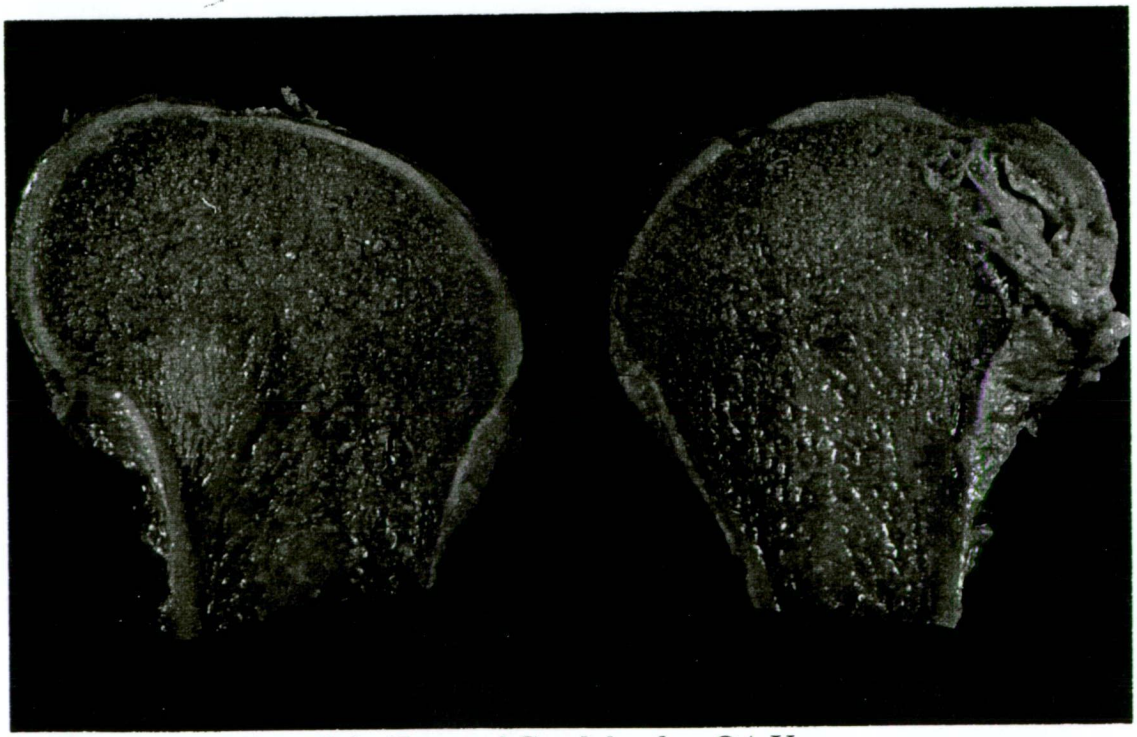


Figure 4-4 Side View of the Femoral Condyle of an OA Knee

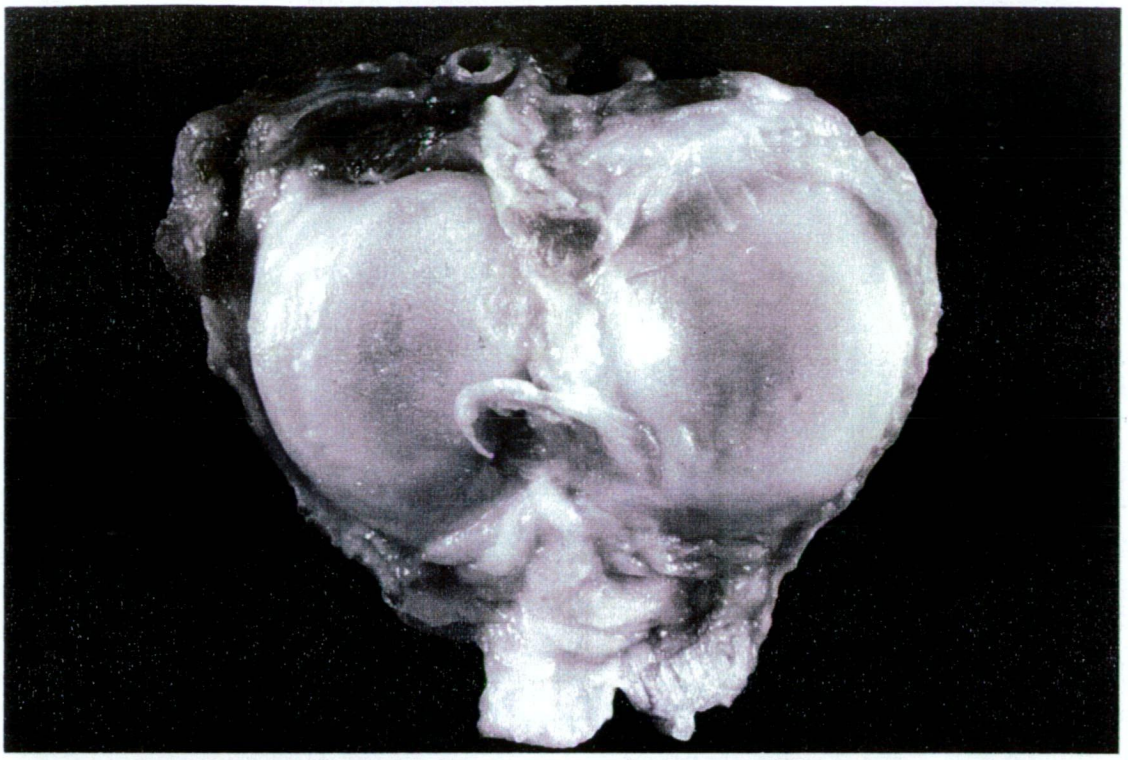


Figure 4-5 Plan View of the Tibial Plateaux of a Normal Knee

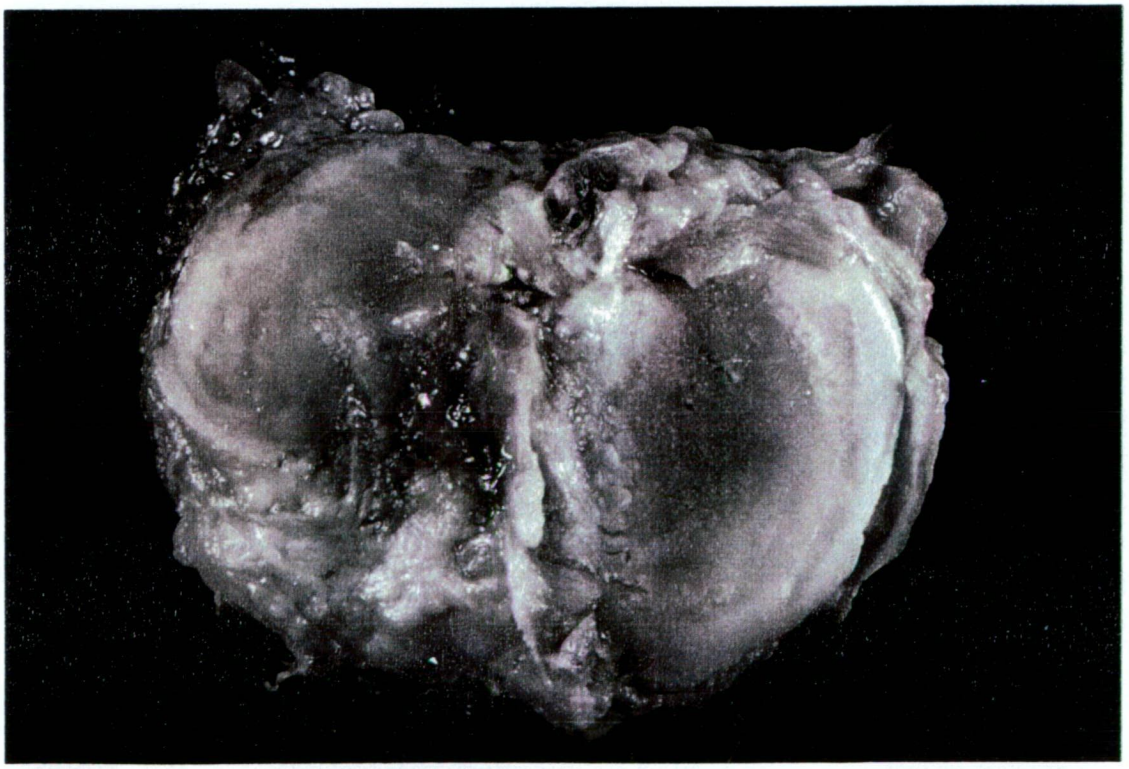


Figure 4-6 PlanView of the Tibial Plateaux of an OA Knee

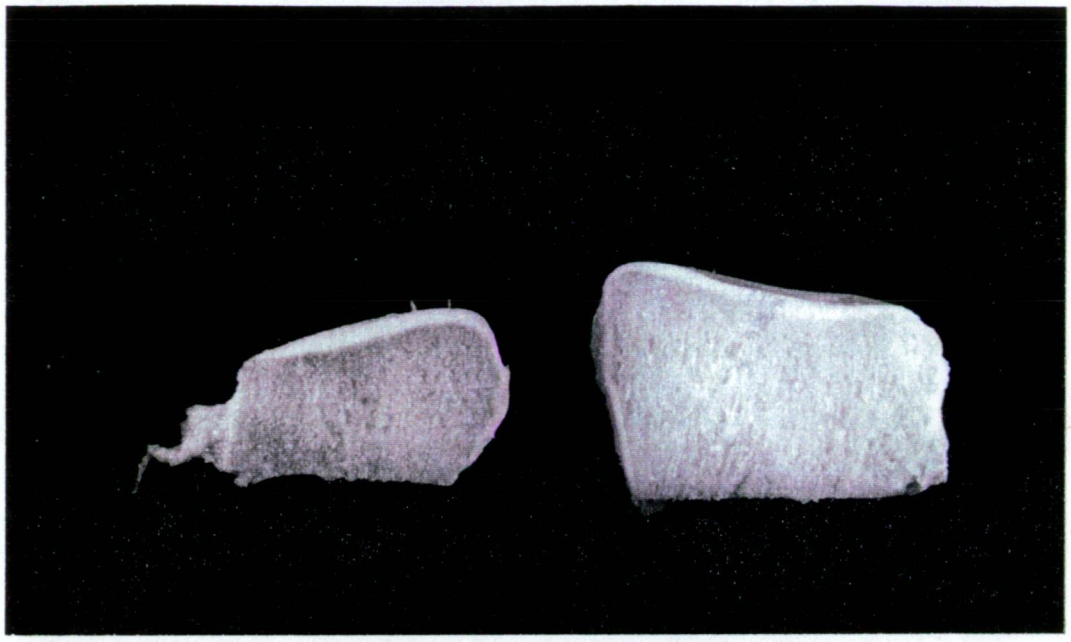


Figure 4-7 Side View of the Tibial Plateaux of a Normal Knee

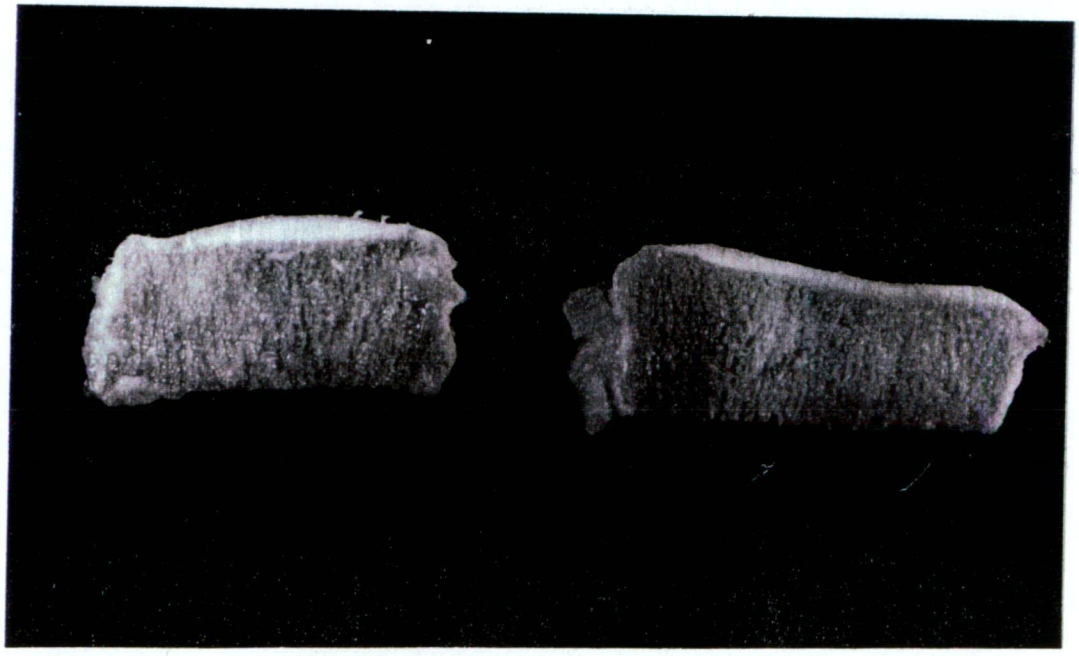


Figure 4-8 Side View of the Tibial Plateaux of an earlyOA Knee

Cartilage softened and slightly transluscent with mild fibrillation

4.3 MICROSCOPIC MORPHOLOGY: CLASSICAL HISTOLOGY

An initial overall assessment was made using H & E stain. From this the cartilage was given an overall histological grading again divided into normals, ageing, early OA, moderate OA and severe OA. In some cases, the macroscopic and histological gradings were different, the microscopic examination frequently progressing the case into a more severe classification. Each joint compartment was given an overall grade on the basis of the anatomical region with the most severe disease. It was noted frequently where there was moderate or severe disease on the weight bearing surface of the femur that there was less severe disease on the posterior non-weight bearing portion. Likewise, where one compartment was affected with established OA the other compartment had early changes (TABLES 4-2 and 4-3).

4.3.1 MATRIX AND CHONDROCYTES

Microscopically the normal arrangement of chondrocytes was seen to become increasingly disrupted with ageing and throughout the successive stages of osteoarthrosis. The normal lacunar arcades of cells that run perpendicular to the articular surface in zones III and IV became disrupted, and the chondrocytes focally hypertrophy with more severe disease. The upper zone chondrocytes, normally ellipsoid and flattened parallel to the surface also hypertrophy and tend to form clones which may consist of up to 20-30 syncytial chondrocytes. Clones tended to "congregate" closer to the margins of clefts and fissures. Overall cellularity appeared to increase, however, there were large areas of acellular cartilage matrix. There were characteristic and distinctive differences between ageing and early osteoarthrosis; in ageing there was less marked disruption of chondrocytes throughout all zones and less clone formation; zone V pegs and the tidemark became disrupted in both, however, changes were more marked in early OA. Distinctive areas of fibrosis were seen on the surface and within cartilage in the severe groups. Figures 4-9 to 4-22 show examples of these features.

4.3.2 CHONDRO-OSSEOUS JUNCTION (COJ)

This section considers the COJ in ageing and OA (The normal anatomy of the tidemark is described in section 4.5). In both the ageing and early OA groups there was disruption of the normal tidemark with tidemark duplication or triplication and early invasion of vessels from the underlying subchondral bone plate. The changes appeared to be much more pronounced in the early OA group. In this group the normal architecture of the tidemark was quite distinctively disrupted whereas in the ageing group it appeared to be maintained or minimally altered. The moderate and severe groups were characterised by increasingly complete disruption of the tidemark region with the formation of numerous tidemarks which in places either merged or became discontinuous. Very pronounced vascular invasion was present and in many places distinctive multinucleated cells chondroclasts could be seen in front of the 'invading' vascular tufts.

Type A, B and C contacts were assessed. The author found these difficult to evaluate from previous descriptions, however, the overall impression was that there was an increasing frequency of contacts with increasing severity of disease. Splits at the tidemark were frequent and seen in all groups, however, they were more clearly defined in the normals, early and ageing groups. In these groups there was a distinctive tidemark relative to the more severe groups where there were multiple tidemarks. It is possible that these splits represent an artefact due to sectioning in relation to a possible weakness of this line. Figures 4-23 to 4-28 show examples of these features.

4.3.3 SUBCHONDRAL BONE PLATE

In this series very little change was seen in the subchondral bone in the normals, ageing and early OA groups. In the moderate and severe groups, a variety of changes were seen in the subchondral bone plate. In places trabecula appeared thinned with occasional cysts, whereas in other areas bone was thickened and the medullary cavity fibrotic. Using the classical stains assessment of osteocytes, osteoblasts and osteoclasts was difficult. There were small fractures in the subchondral bone, however, these were infrequently seen possibly reflecting the relatively gentle

decalcification procedures which may reduce artefactual fractures on sectioning. In the most severe groups, with total loss of cartilage, there was distinctive thickening of the subchondral bone plate, which took on an extremely sclerotic appearance. Occasional foci of new bone, woven bone, endochondral ossification and fibrosis were seen. Fat necrosis and the presence of multinucleated giant cells as described in severe disease were seen infrequently. A summary of these findings is provided in Table 4-2. Figure 4-29 and 4-30 show examples of these features.

Table 4-3 on pages 123-124 shows the overall histological grading of different knee compartments, anatomical sites and includes macroscopic and clinical assessment. The abbreviations used in these tables are:

Code – case sample number

1M/1L – medial/lateral tibial plateaux

2 – intercondylar notch

3M/3L – inferior medial and lateral femoral condyles

4M/4L – posterior medial and lateral femoral condyles

Grade - overall grade for the compartment

Macro – visual assessment grade

Clinical – clinical assessment grade

Table 4-2 Summary of Classical Histology – Haematoxylin and Eosin

CARTILAGE GRADE	FEATURES OF STAINING
NORMAL	Smooth surface, Matrix uniform, pale eosinophilic Zones I-III Zone IV more eosinophilic. Zone V strongly eosinophilic. Chondrocytes- round, in arcades in deep zones Elliptical, flat in upper zones. Pale cytoplasm, eosinophilic membrane. Tidemark present -single line. Osteocytes stain, Thin sub-chondral plate. Vascular endothelium stains. Sub chondral bone strongly eosinophilic.
AGED	Surface- some superficial fibrillation. Matrix- Uniform pale eosinophilic I-IV, Zone V-moderately eosinophilic. Architecture disrupted zones I and II. Chondrocytes- small clones. Tidemark- occasional duplication and vascular invasion. Osteocytes unchanged.
EARLY OA	Surface smooth but indents. Matrix – similar to ageing. Chondrocytes – marked alteration in architecture. Clones and frequent small clones zones I and II. Tidemark duplication and triplication. Marked vascular invasion.
MILD OA	Surface- fissuring and early fragmentation-loss of smooth surface. Matrix- Less uniform staining, acellular, chondrolysis. Chondrocytes- loss of arcades, formation of clones. Gen. disorganised histopathology, pleomorphic, hypertrophic Tidemark -duplication sometimes multiple. Vascular contacts and occasional neovascularisation. Further sub-chondral plate expansion, bone vascularisation.
SEVERE OA	Surface -deep fissures & defects, loss of cartilage. Matrix – pale, acellular, fibrosis, chondrolysis. Chondrocytes- Hypertrophic+prominent nuclei, dysmorphic, Clones present ++. Tidemark disorganised, multiple duplications. Neovascularisation into non calcified cartilage. Bony eburnation, Further thickening sub-chondral plate. sub-chondral bone cysts. New cartilage.

Table 4-3 Histological Grading of Different Knee Sites [Normal (N), Early, Moderate, Severe OA (E,M,S), Uni-/bilateral OA (U,B)]

CODE	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1M	N	S	X	N	A	S	M	A	S	A	A	E	S	S
3M	E	M	S	N	A	S	M	A	E	N	N	M	E	S
4M	E	S	X	N	A	E	E	A	M	N	A	S	X	M
GRADE	E	S	X	N	A	M	M	A	M	E	N	S	S	S
2	E	X	M	N	A	M	S	S	E	A	N	M	E	M
GRADE	E	X	M	N	A	M	M	S	E	E	N	M	E	M
1L	M	M	M	N	M	M	M	A	M	E	N	E	E	E
3L	E	E	N	N	M	A	E	A	X	N	A	X	E	E
4L	E	M	N	N	M	M	E	A	M	N	A	M	E	E
GRADE	M	M	MO	N	M	M	E	A	S	A	A	E	E	E
MACRO	E/M	S/M	X/M	N/N	M	S/M	M/M	A/A	M/S	E	A/A	S/E	S/E	S/E
CLINICAL	B	U	B	N	N	B	U	N	U	N	N	U	U	B

CODE	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1M	N	N	A	S	S	E	M	S	M	S	M	S	S	S
3M	N	N	A	S	S	E	M	S	S	M	M	S	S	S
4M	N	N	A	M	S	E	S	S	M	M	M	M	M	S
GRADE	N	N	E	S	S	E	S	S	S	S	M	S	S	S
2	N	N	M	E	X	S	M	E	E	M	E	E	M	M
GRADE	N	N	M	E	X	S	M	E	E	M	E	E	M	M
1L	N	N	N	E	E	S	M	S	E	E	E	M	S	E
3L	N	N	A	E	M	X	M	E	E	M	E	E	M	E
4L	N	N	A	E	E	S	X	E	S	E	E	M	M	E
GRADE	N	N	A	E	E	S	M	S	E	M	E	M	S	E
MACRO	N/N	N/N	A/A	S/E	S/E	E/S	S/M	S/E	S/S	S/M	M/E	S/M	S/S	S/M
CLINICAL	N	N	N	U	U	U	B	B	B	B	U	B	B	B

CODE	29	30	31	32	33	34	35	36	37	38	39	40	41	42
1M	E	M	S	M	M	M	M	M	S	S	M	S	S	S
3M	M	M	M	S	M	X	S	E	M	M	X	S	E	S
4M	E	M	S	M	M	X	E	M	S	X	X	S	X	X
GRADE	M	M	S	S	M	M	S	M	S	S	M	S	S	S
2	E	X	M	S	M	X	S	E	M	M	X	M	S	E
GRADE	E	M	S	S	M	E	S	E	M	M	X	M	M	E
1L	M	M	M	M	M	M	M	M	E	E	M	E	M	M
3L	E	M	E	S	E	M	E	M	M	E	X	M	M	S
4L	E	E	E	S	M	X	M	E	E	M	X	E	X	M
GRADE	M	M	M	S	M	M	M	M	M	M	M	M	M	S
MACRO	M/M	M/M	S/M	S/S	M/M	M/M	S/M	M/M	S/M	S/M	M/M	S/M	M/M	S/S
CLINICAL	B	B	B	B	B	B	B	U	B	B	B	B	B	B

CODE	43	44	45	46	47
1M	S	S	E	E	S
3M	S	M	E	E	S
4M	S	M	E	E	S
GRADE	S	S	E	E	S
2	E	M	E	E	S
GRADE	E	M	E	E	S
1L	E	S	S	S	S
3L	M	M	S	S	S
4L	M	M	S	S	S
GRADE	M	S	S	S	S
MACRO	S/M	S/S	E/S	E/S	S/S
CLINICAL	B	B	U	U	B

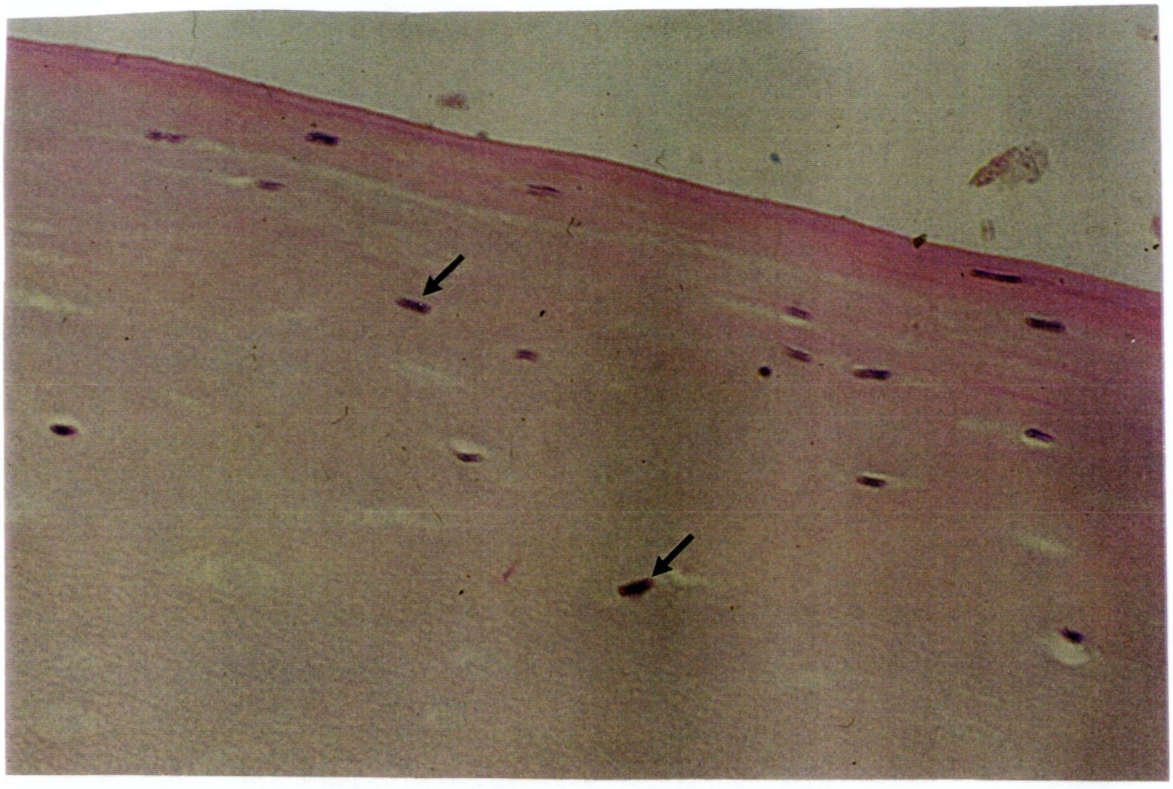


Figure 4-9 Normal Articular Cartilage Surface (H & E)

Smooth surface, ellipsoidal zone I cells parallel to surface and more rounded zone II cells. The arrows point to these cells in zones I and II.

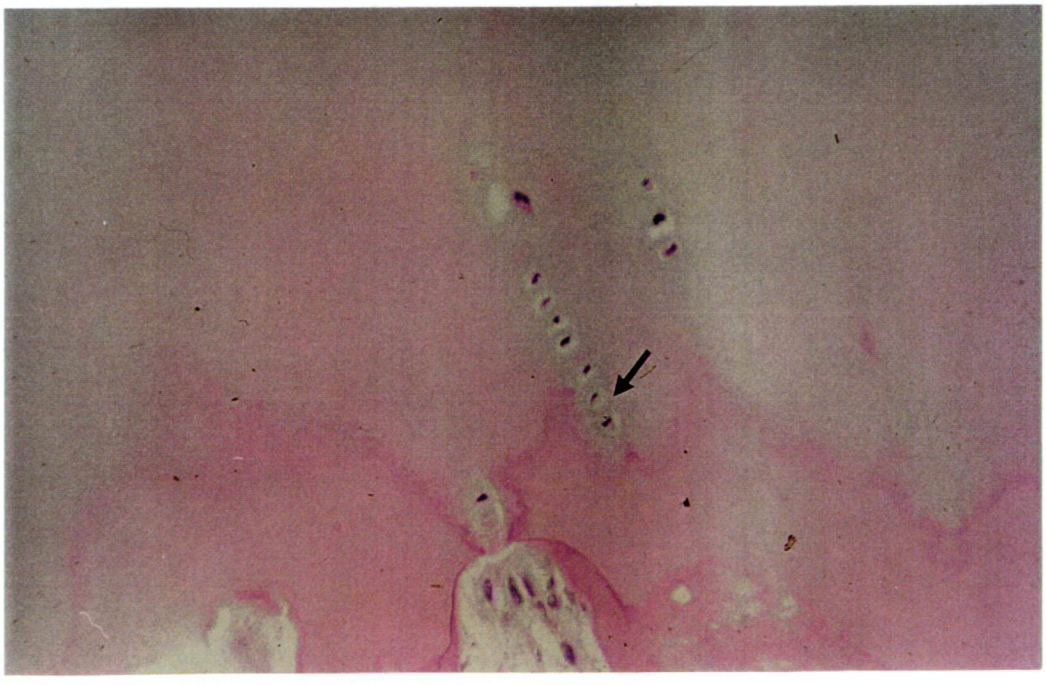


Figure 4-10 Normal Articular Cartilage (H & E)

Zone IV and chondro-osseous junction. Columns of cells seen extending to a single tidemark with calcified and uncalcified cartilage. The arrow points to these columns.

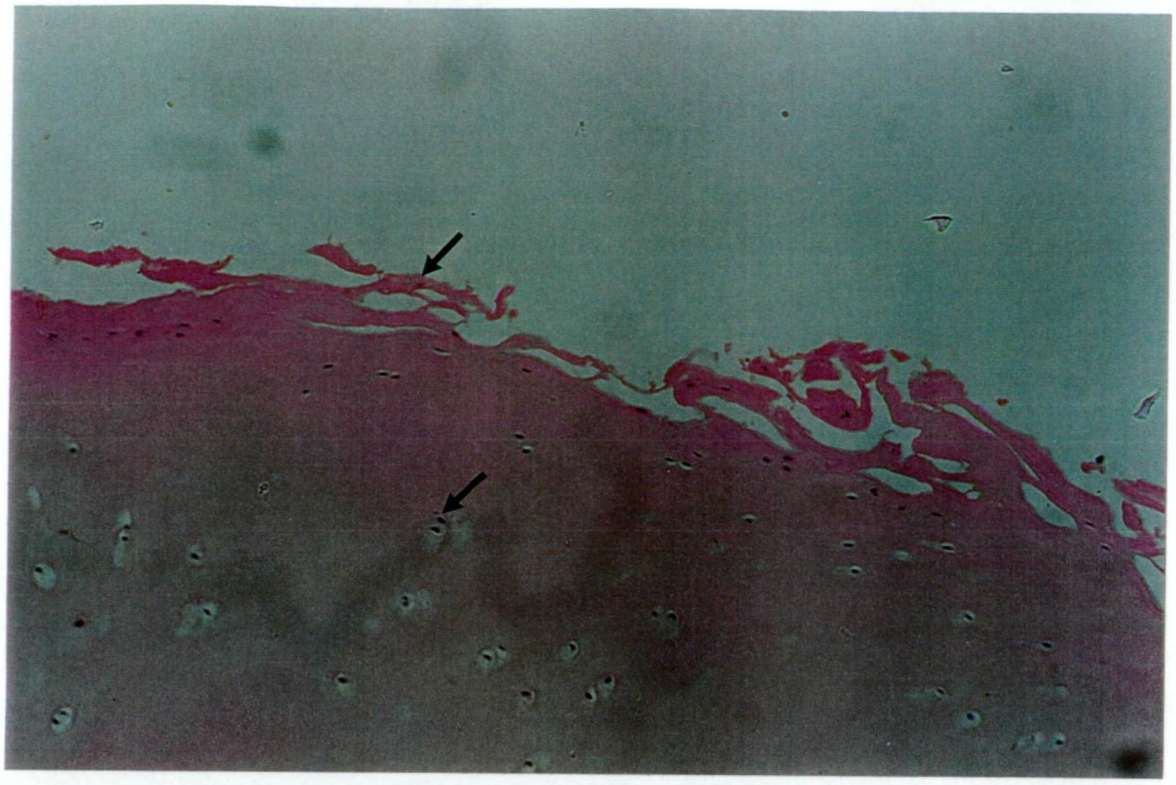


Figure 4-11 Ageing Articular Cartilage Surface (H & E)

Very superficial fibrillation, loss of normal chondrocytes and formation of small clones in zones I and II. The arrow points to the surface and small clones.

Figure 4-12 Ageing Articular Cartilage Zone IV and Chondro-osseous Junction (H & E)

Some disruption of normal columns of cells in zone IV. Early duplication/thickening of the tidemark and horizontal tidemark splits. The arrow points to the tidemark region.

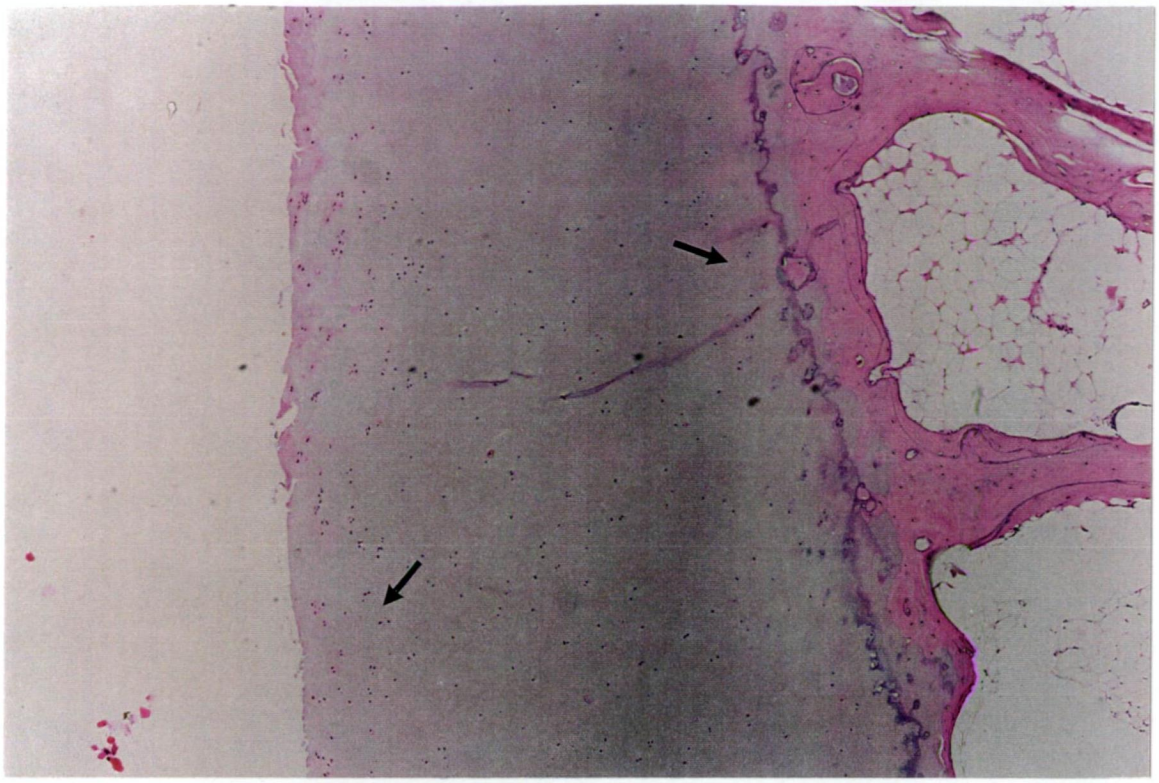


Figure 4-13 Early OA Full Thickness (H & E)

Minimal disruption of surface and altered chondrocyte architecture with formation of prominent clones (Arrow). Chondrocyte architecture is altered in zones III and IV with loss of columns and there is early tidemark duplication (Arrow).

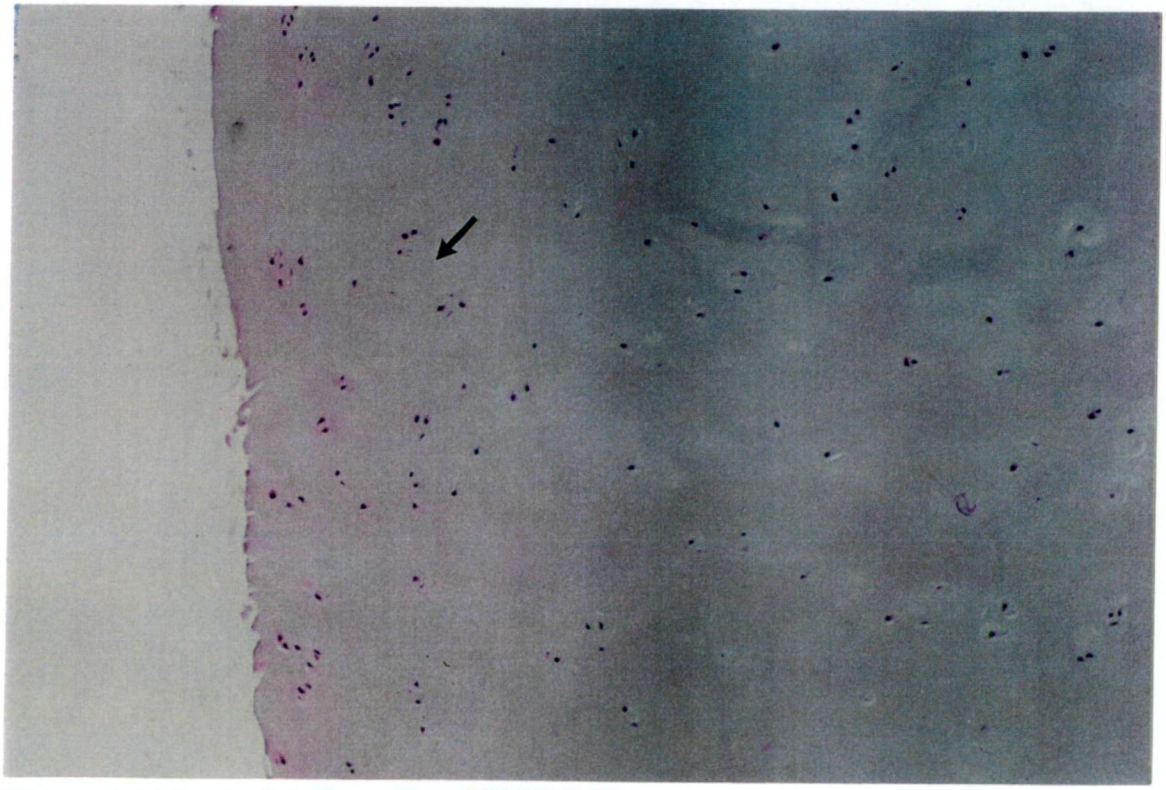


Figure 4-14 Early OA Zones I and II (H & E)

Surface shows minimal fibrillation. The chondrocyte architecture is disrupted with areas of acellular matrix and prominent small clones (up to four chondroctyes). The arrow points to the small clones in an acellular area.

Figure 4-15 Moderate OA zones I and II (H & E)

Moderate surface fibrillation and marked disruption of chondrocytes with formation of medium sized clones (up to 8 chondrocytes). Arrow points to a clone.

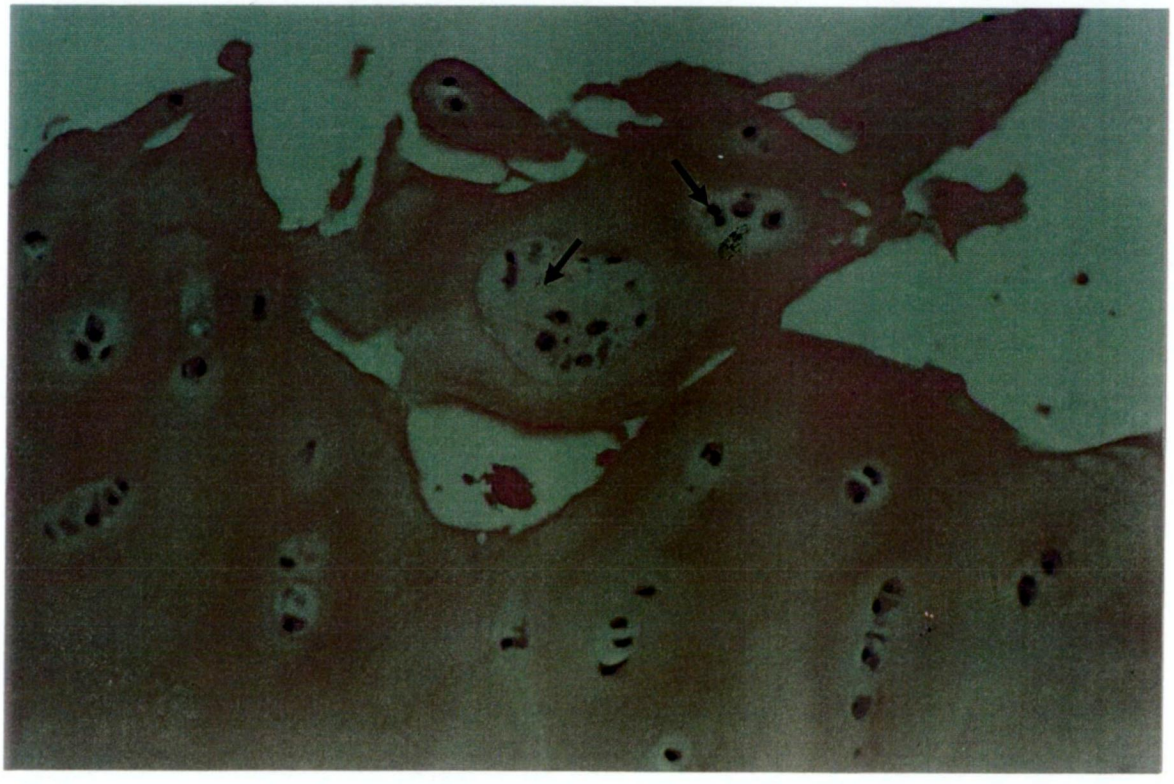


Figure 4-16 Moderate OA High Power View of Zone I (H & E)

Areas of fibrillation with prominent clone formation. Arrows point to prominent clones with several nuclei and pallor staining matrix.

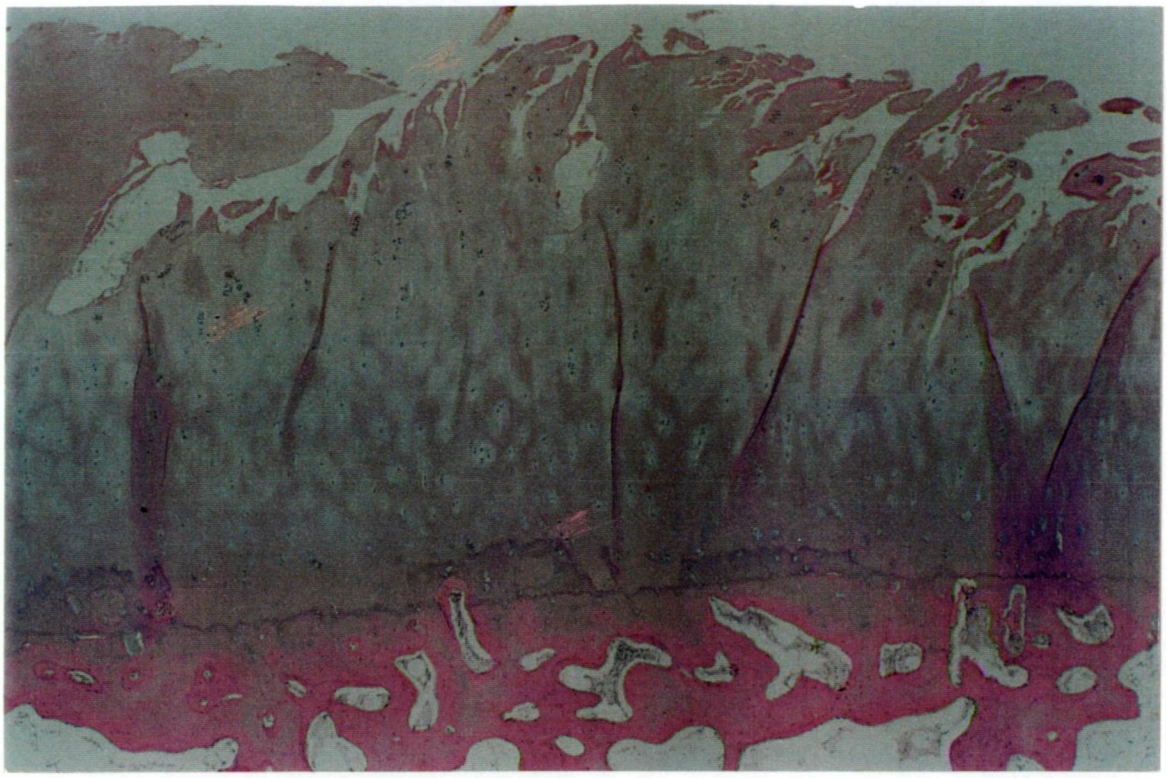


Figure 4-17 Severe OA Full Thickness (H & E)

Very marked fibrillation, prominent clone formation and disruption of overall architecture.

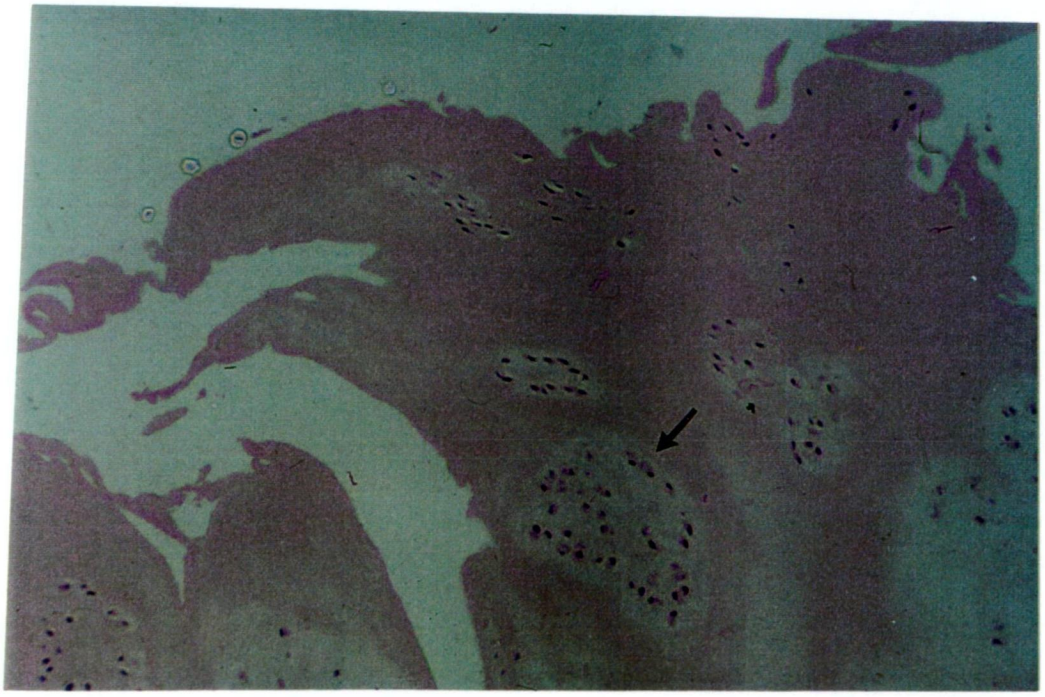


Figure 4-18 Severe OA High Power View Zone I (H & E)

Massive chondrocyte clones (up to 30 chondrocytes) seen in fibrillated surface zone. Arrow points to large pale staining clone.

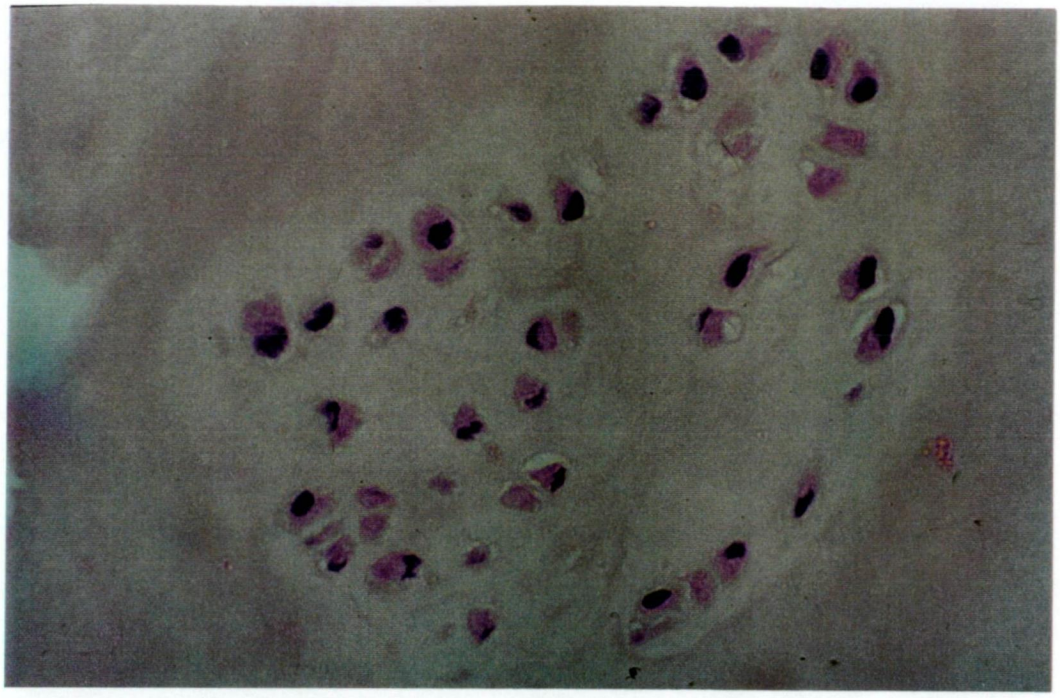


Figure 4-19 Large Chondrocyte Clone in Severe OA (H & E)

Chondrocyte clone comprised of approximately 30 chondrocytes.

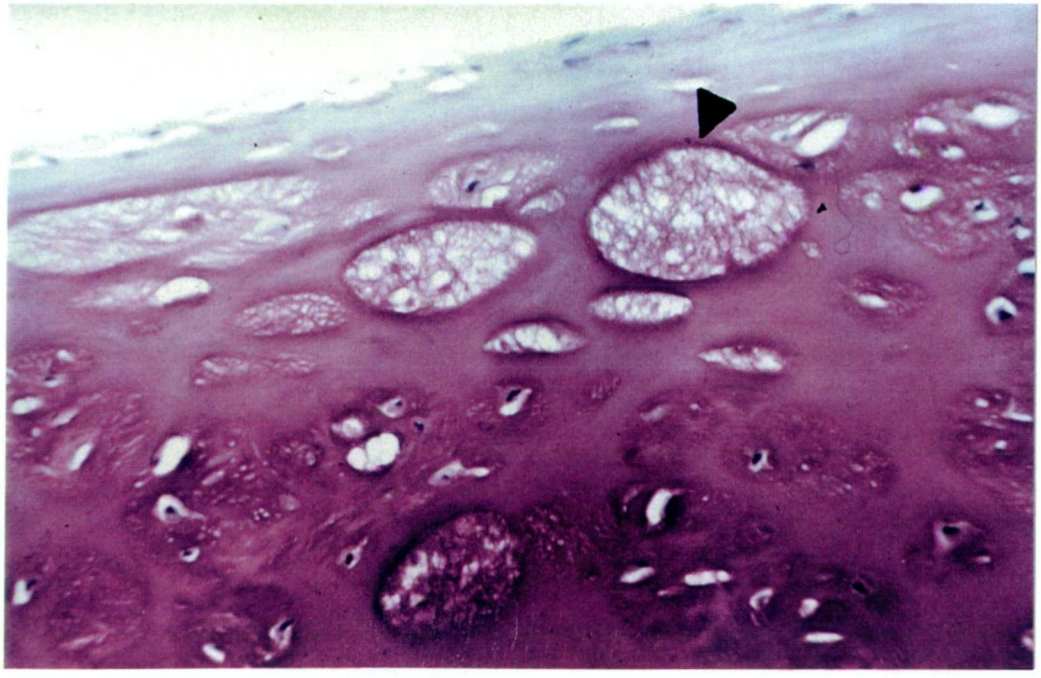


Figure 4-20 Hypertrophic Chondrocytes Early OA (Toluidine Blue)

Arrow points to large forming hypertrophic chondrocyte in zone I.

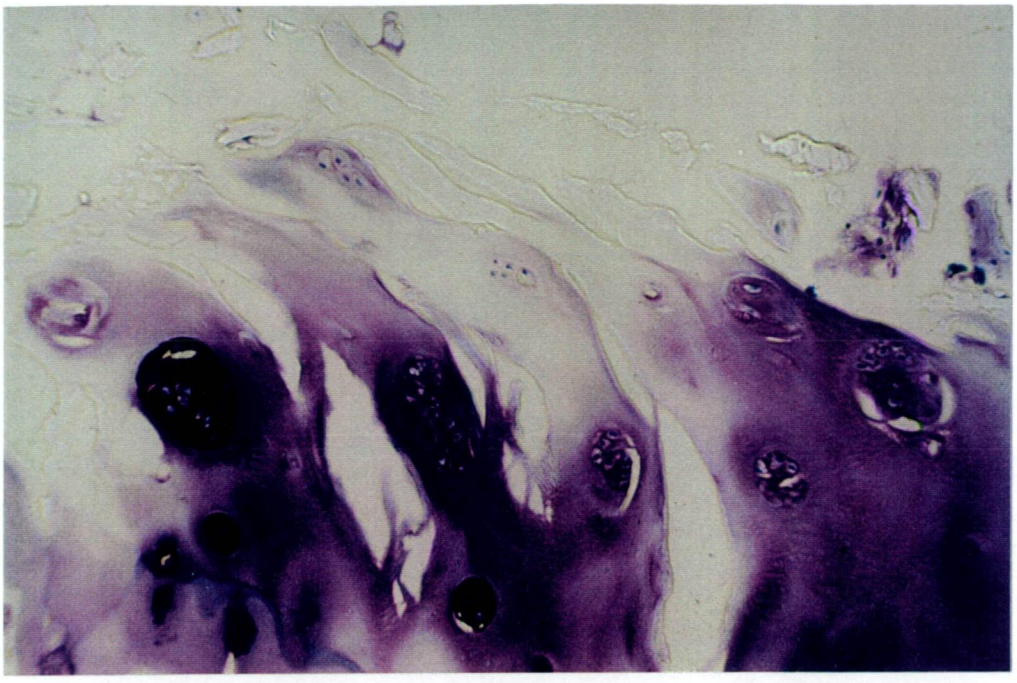


Figure 4-21 Fibrillation in Severe OA (Toluidine Blue)

Prominent fibrillation in severe OA with areas of variable staining matrix and clones.

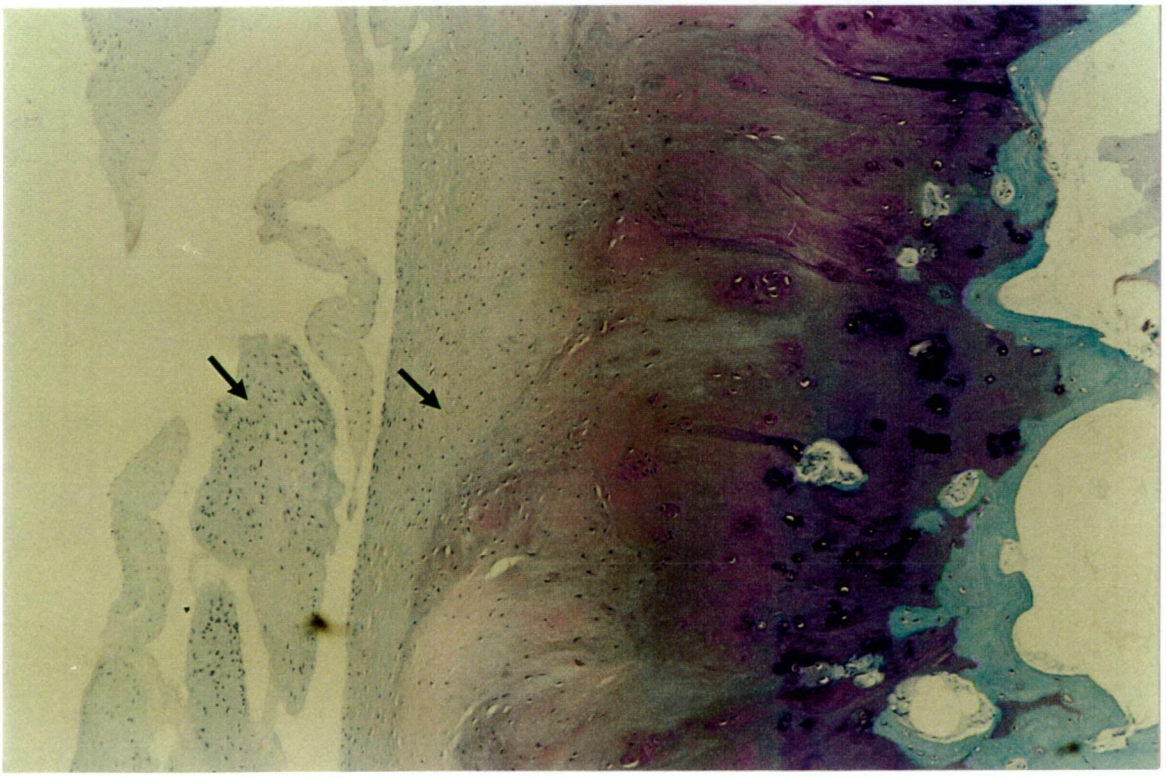


Figure 4-22 Fibrosis in Severe OA (Toluidine Blue)

Area of superficial and intramatrical fibrosis adjacent to an area of fibrillated cartilage in severe OA. Arrows point to these areas of fibrosis.

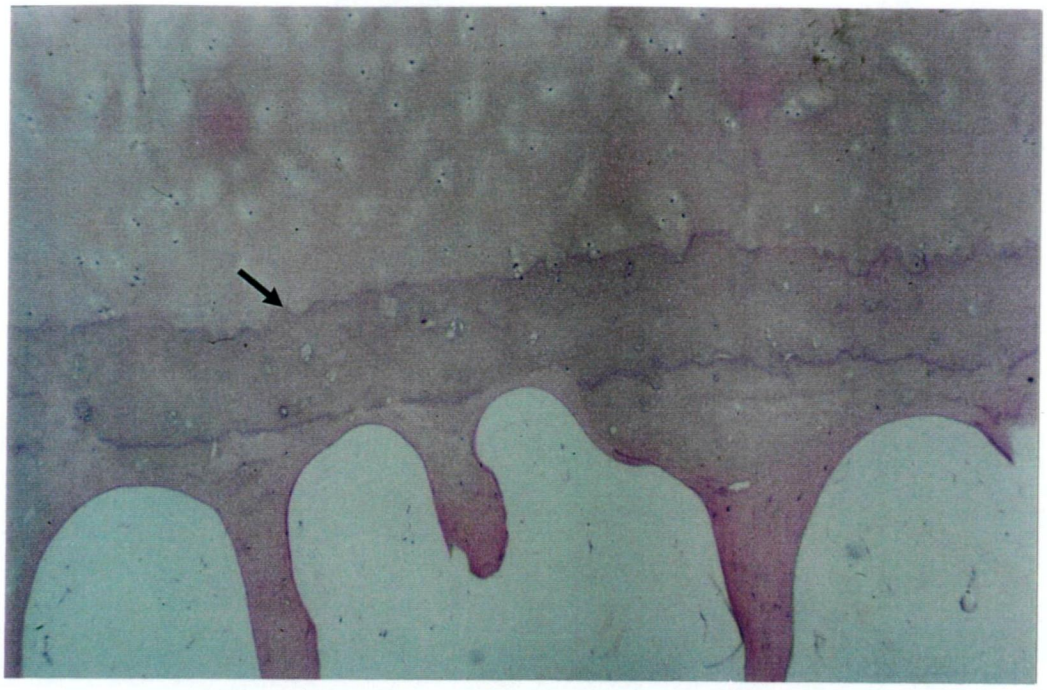


Figure 4-23 Tidemark Duplication in Ageing (H & E)

Prominent separation of two tidemarks in ageing. Arrow points to upper of two tidemarks.

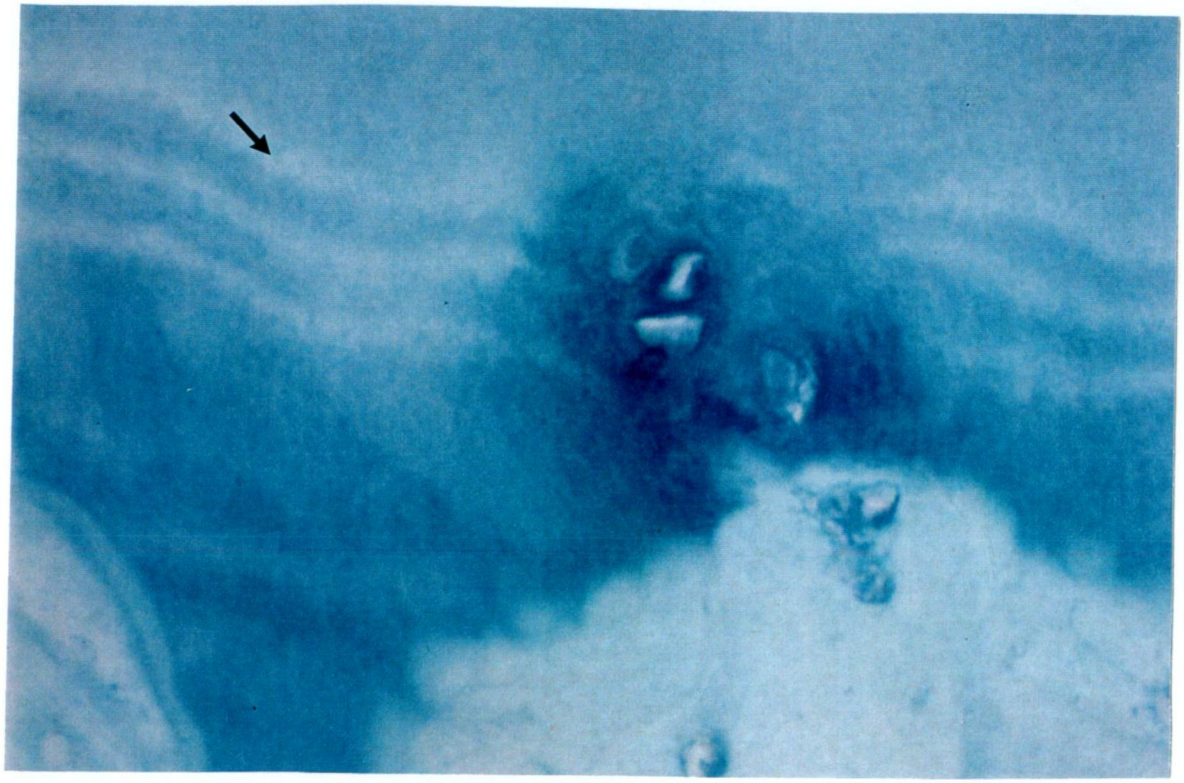


Figure 4-24 Tidemark Triplication in Early OA (Alcian Blue)

Prominent triplication of the tidemark (no staining) in early OA stained with Alcian Blue stain. Arrow points to uppermost of three tidemarks.

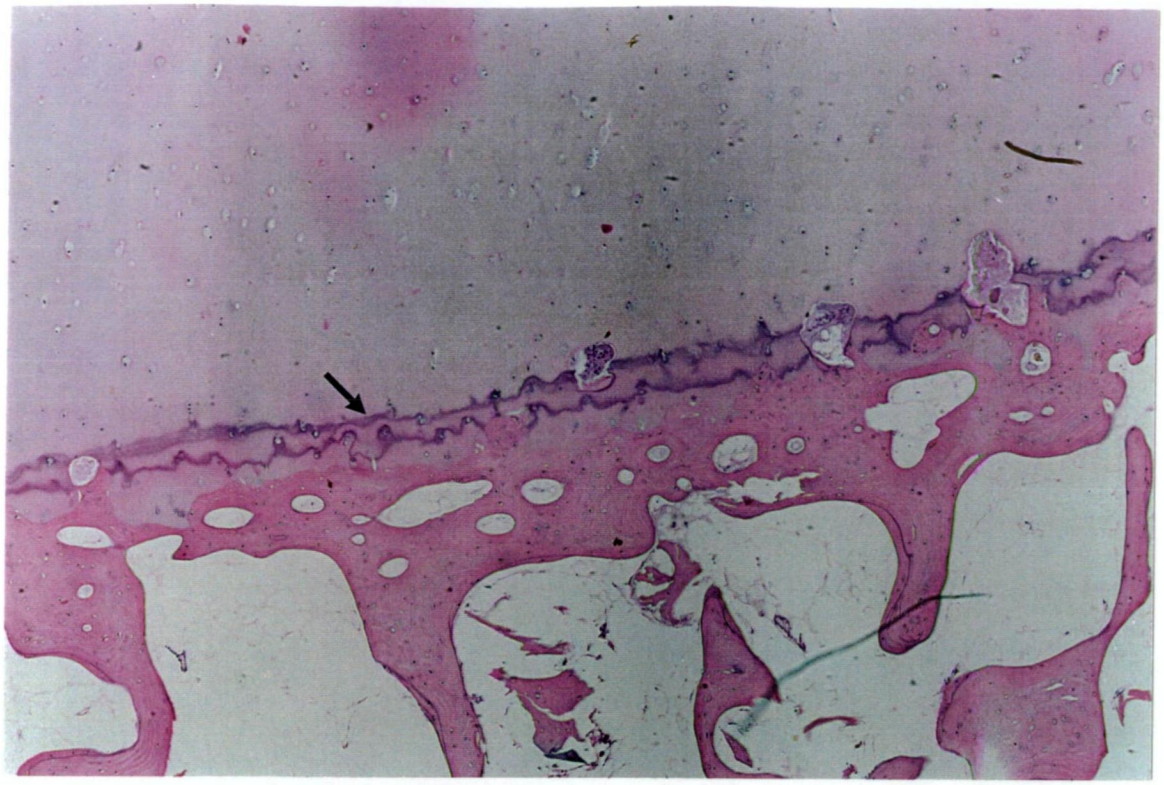


Figure 4-25 Tidemark Duplication and Vascular Invasion in Early OA (H & E)

Tidemark shows duplication with vascular plugs extending across tidemark into uncalcified cartilage, these are tipped by chondrocytes. Arrow points to uppermost tidemark.

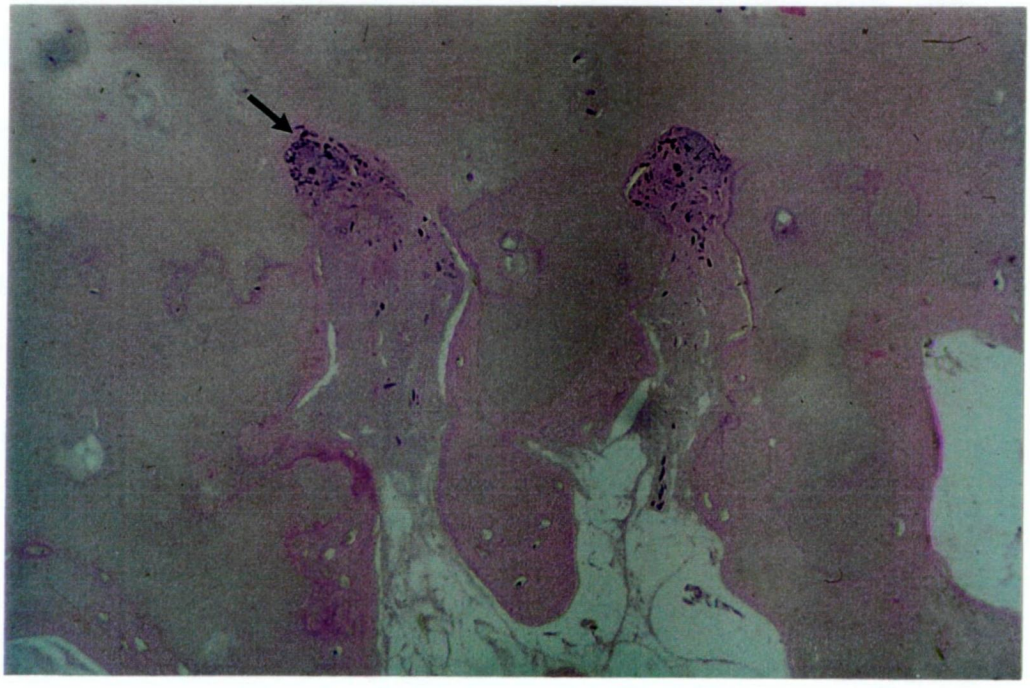


Figure 4-26 Vascular Invasion in Moderate OA (H & E)

Prominent vascular plugs extending from marrow through calcified cartilage across tidemark into uncalcified cartilage. Plugs composed of vessels and tip contains multinucleated chondroclasts. Arrow points to tip of "invading" vascular plug.

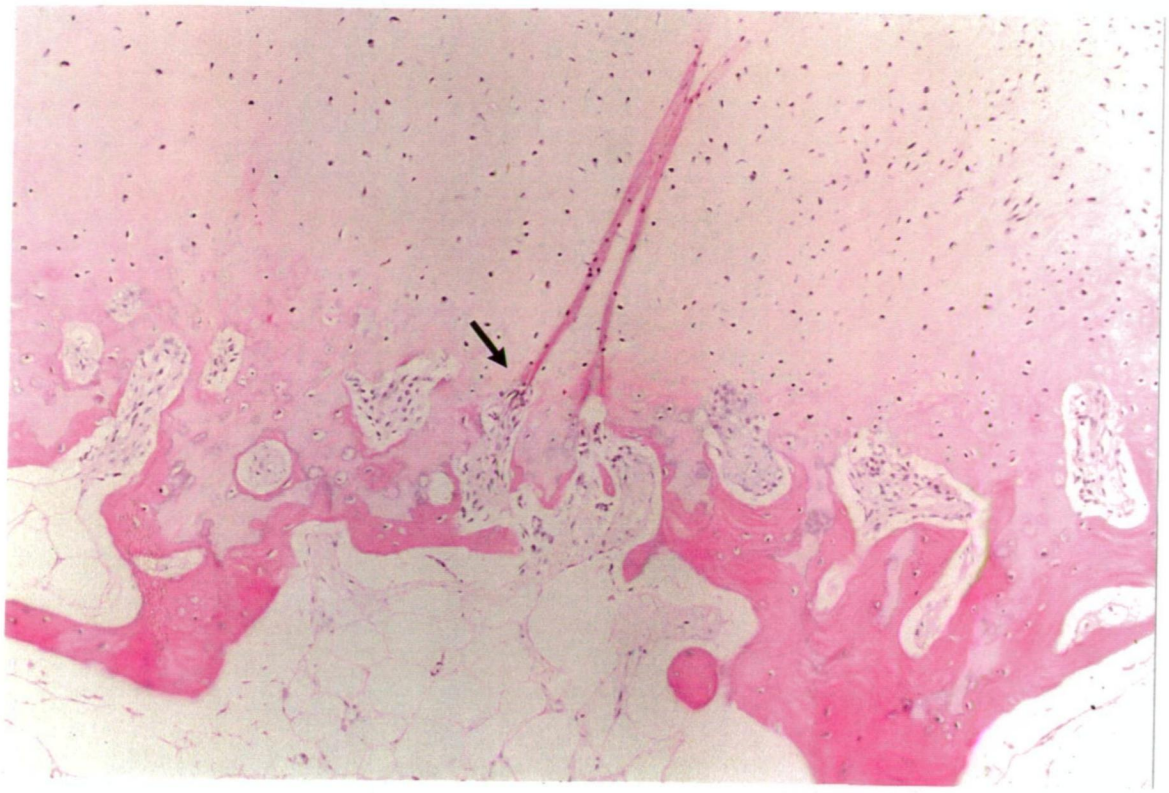


Figure 4-27 Chondro-Osseous Junction in Severe OA (H & E)

Complete disruption of the chondro-osseous region with abnormal chondrocyte morphology in zone IV and prominent vascular invasion. Arrow points to disrupted tidemark region.

Figure 4-28 Tidemark Splits Early OA (H & E)

Horizontal splits at the tidemark in early OA but seen in all groups. Arrows point to splits.

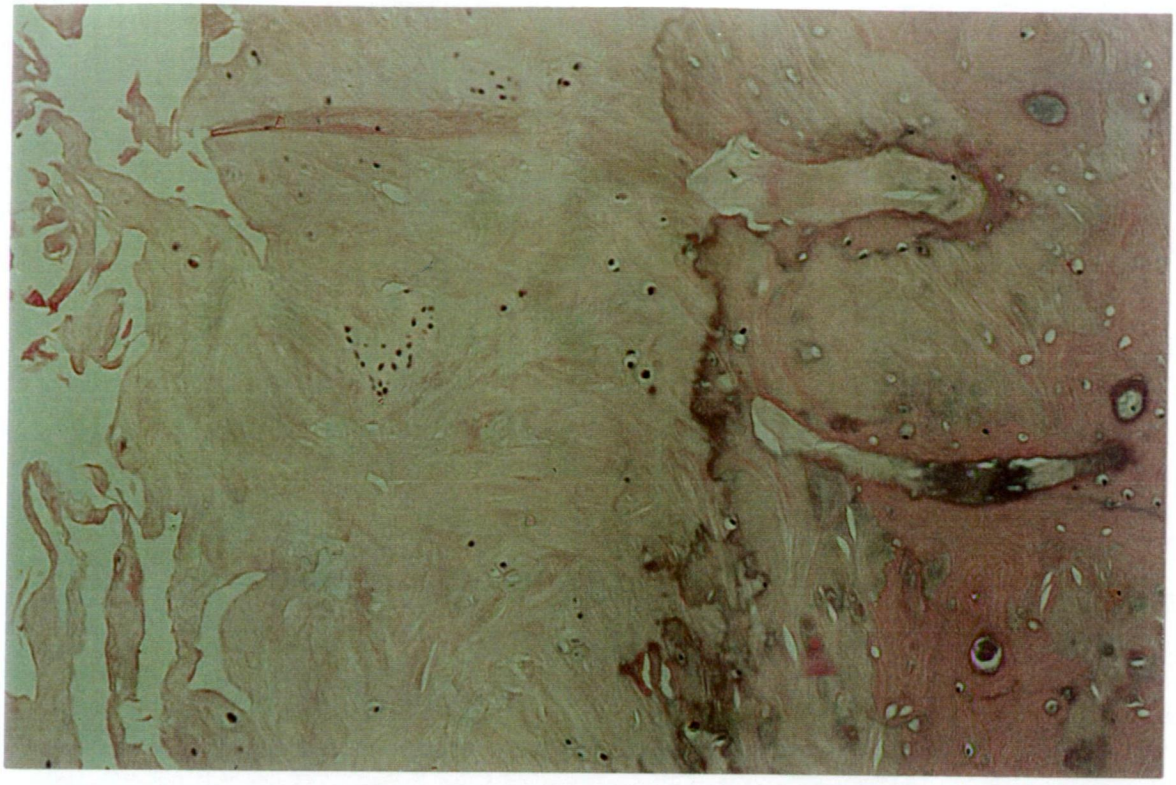


Figure 4-29 Full Thickness Fibrotic Cartilage Severe OA (H & E)

Very fibrotic cartilage in severe OA which is extensively acellular apart from a few clones There is some tidemark duplication and the underlying bone is sclerotic.

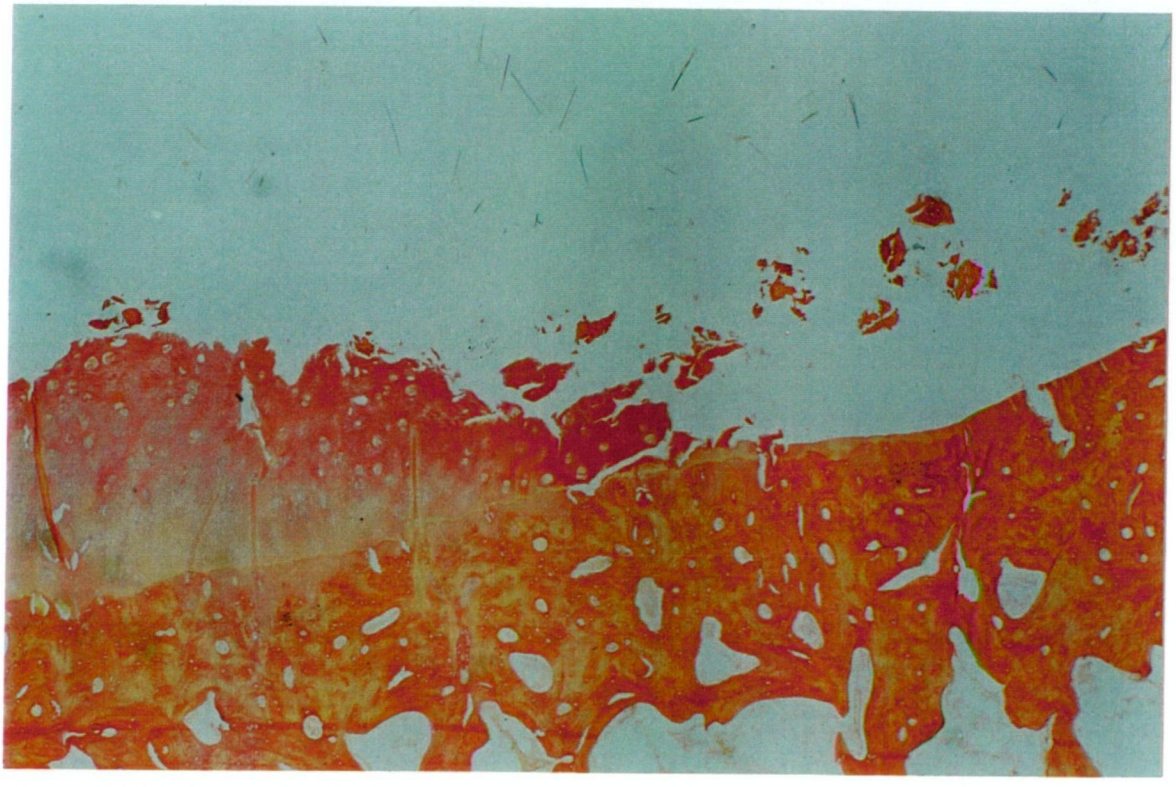


Figure 4-30 Eburnation in Severe OA (PSR)

Full thickness cartilage loss with underlying eburnated sclerotic bone in severe OA.

Also shows variable collagen staining of bone and matrix with PSR.

4.4 MICROSCOPIC MORPHOLOGY: HISTOCHEMICAL STAINS

4.4.1 GLYCOSAMINOGLYCAN STAINS

Toluidine blue and safranin O revealed generalised reduction of glycosaminoglycans with ageing and progressive OA. In the ageing and early OA groups this was quite noticeable in the superficial zones I and II and was more pronounced in the early OA groups. In the deeper zones there was a gradual decrease in staining intensity across the regions PC>TM>IM. Staining with alcian blue using varying electrolyte concentrations demonstrated the usual staining pattern for chondroitin and keratan sulphate in the normals. That was with relatively more chondroitin sulphate in the middle regions and slightly more keratan sulphate in the deeper regions. As the disease process evolved there appeared to be an increasing intensity of chondroitin sulphate in the pericellular areas and slightly more keratan sulphate in the deeper regions. Toluidine blue stained the tidemark a deep navy blue and safranin O an orange colour. There was no staining with alcian blue. There was light staining of Zone V and virtually no staining of the bone matrix. The cartilage pegs were very clearly delineated from the Zone V cartilage. With all stains there was very variable staining in the severe groups; there were patchy areas of pallor in acellular regions and more deeply stained areas in the regions of the chondrocyte clones. The periclonal and intraclonal matrix often stained very deeply.

A summary of these findings is provided in Table 4-4.

Figures 4-31 to 4-36 show examples of the key features.

Table 4-4 Summary of Glycosaminoglycan Stains – Toluidine Blue, Safranin O, Alcian Blue

CARTILAGE GRADE	FEATURES OF STAINING
NORMAL	No surface stain. Matrix- Strong staining Zones IV>III>II esp. pericellular. Zone V Minimal stain. Minimal ZoneI. Chondrocytes- stain strongly, Zones IV>III>II Zone V mild Tidemark - stains. vascular structures - mild staining. Bone - no staining. Osteocytes- nuclei stain.
AGED	Similar pattern to normal. Matrix - sl. less staining ZoneIV but pericellular maintained.
EARLY OA	Similar to aged, more pronounced I and II. Some duplication of tidemark. Zone V- increased staining of chondrocytes and matrix.
MILD OA	General reduction in staining . Relatively retained deeper zones. More loss Zones III-I. Clones stain. Pericellular staining maintained Zone V, IV, III.
SEVERE OA	Surface - loss of stain from Zones III, II (no zone I present.) Retained mild matrix stain Zone V>IV. Most staining cytoplasm and pericellular. Clones stain well. Multiple Tidemark reduplication.

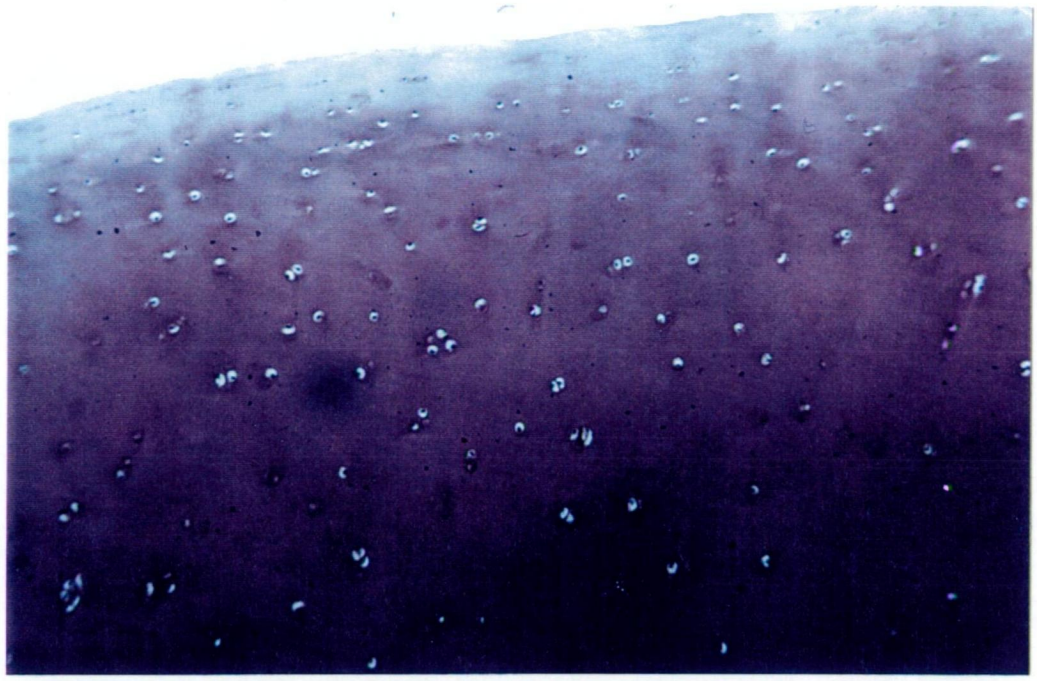


Figure 4-31 Zone I-III in normal cartilage (Toluidine Blue)

Mild loss of staining was seen in the superficial zones with Toluidine Blue stain for glycosaminoglycans.

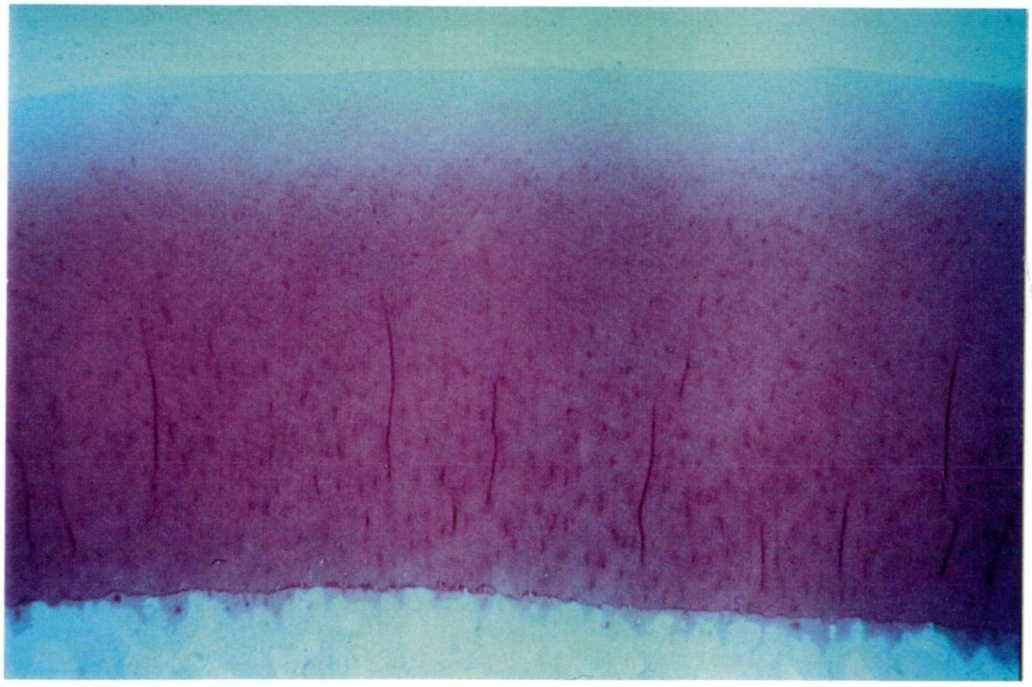


Figure 4-32 Full Thickness Normal Cartilage (SO)

Mild loss of staining was seen in the superficial zone with Safranin O stain for glycosaminoglycans.

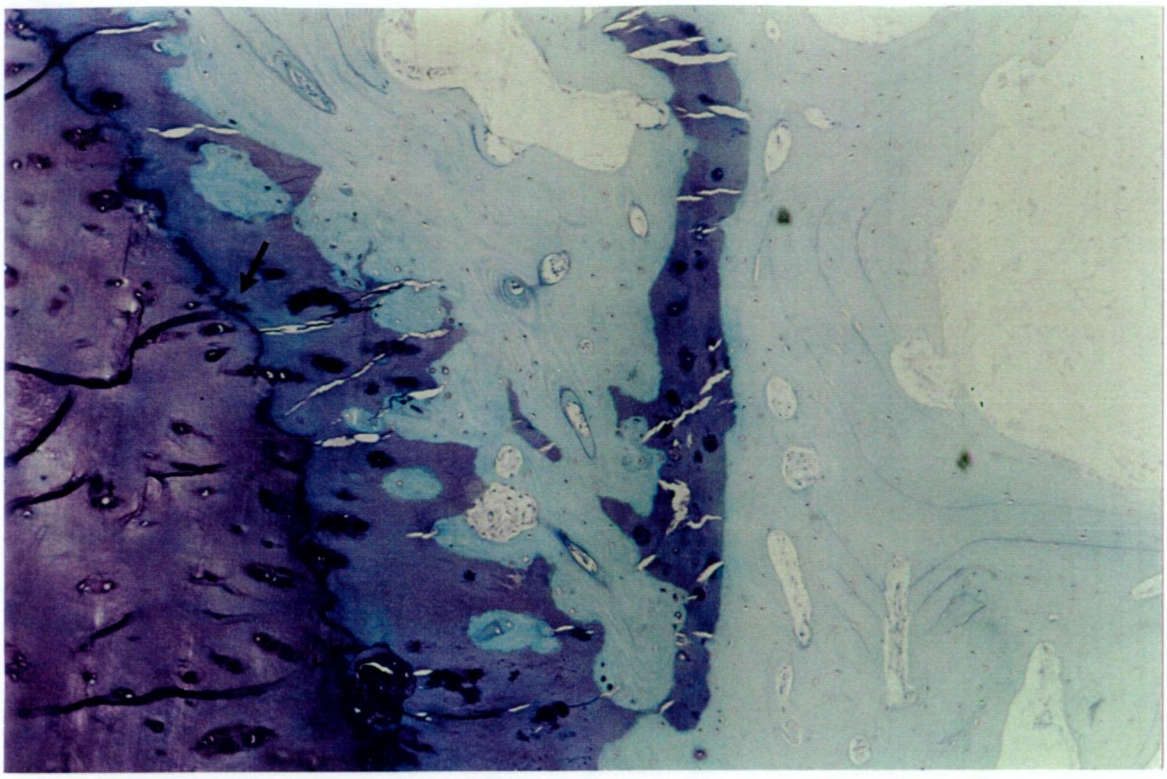


Figure 4-33 Chondro-Osseous Region in Normal Cartilage (Toluidine Blue)

Toluidine Blue stain shows prominent tidemark staining. There is a decrease in the intensity of staining across uncalcified cartilage, calcified cartilage and subchondral bone.

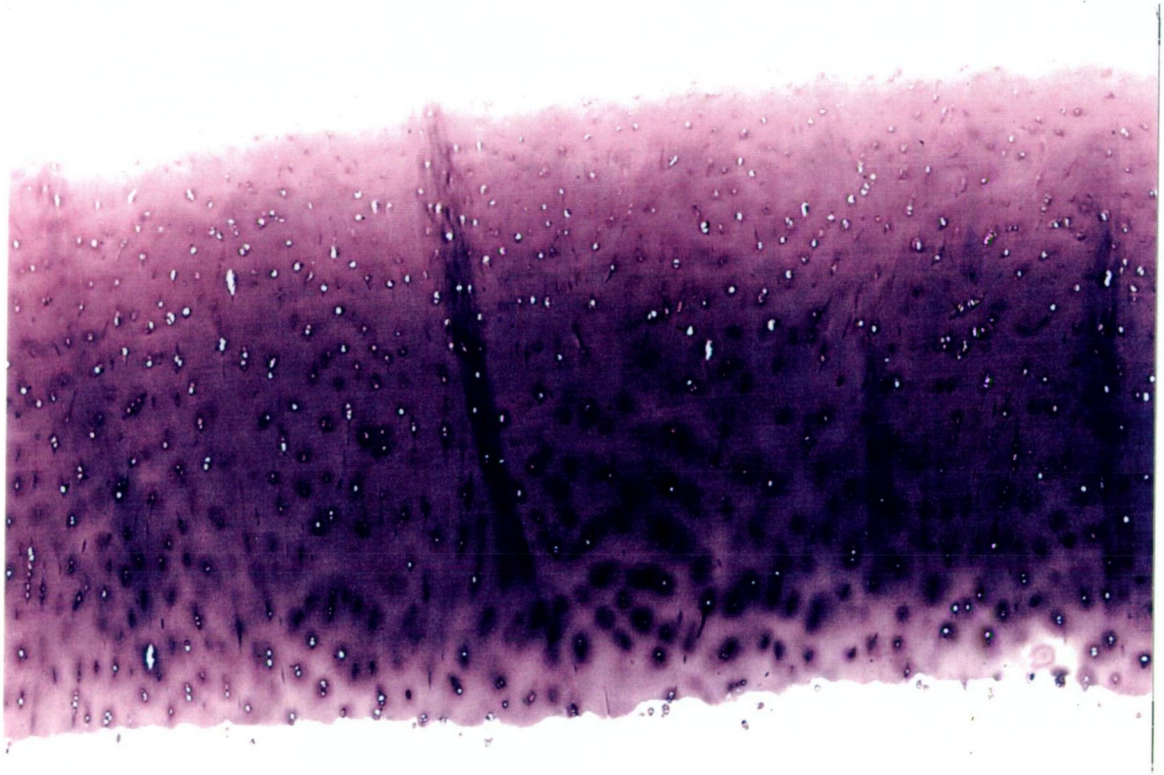


Figure 4-34 Ageing Full Thickness (Toluidine Blue)

Greater loss of glycosaminoglycan staining is seen in the superficial zones and interterritorial matrix of zones III and IV than with the normal group.

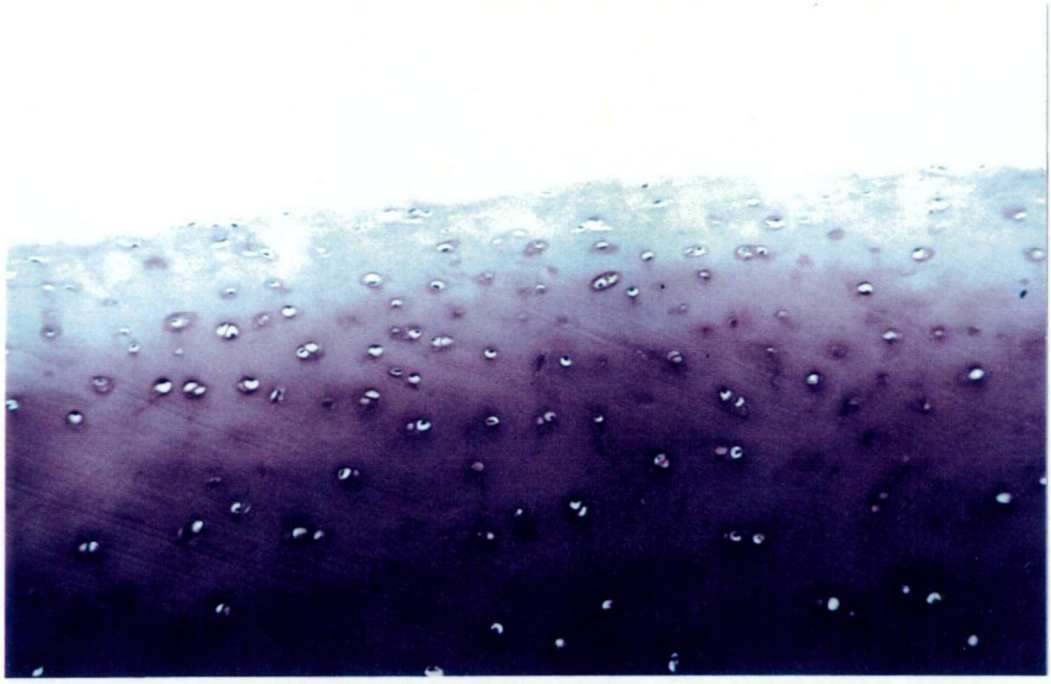


Figure 4-35 EOA Upper Zones (Toluidine Blue)

Marked loss of glycosaminoglycan staining with Toluidine Blue is noted in zones I and II in early OA, slightly more so than the ageing group.

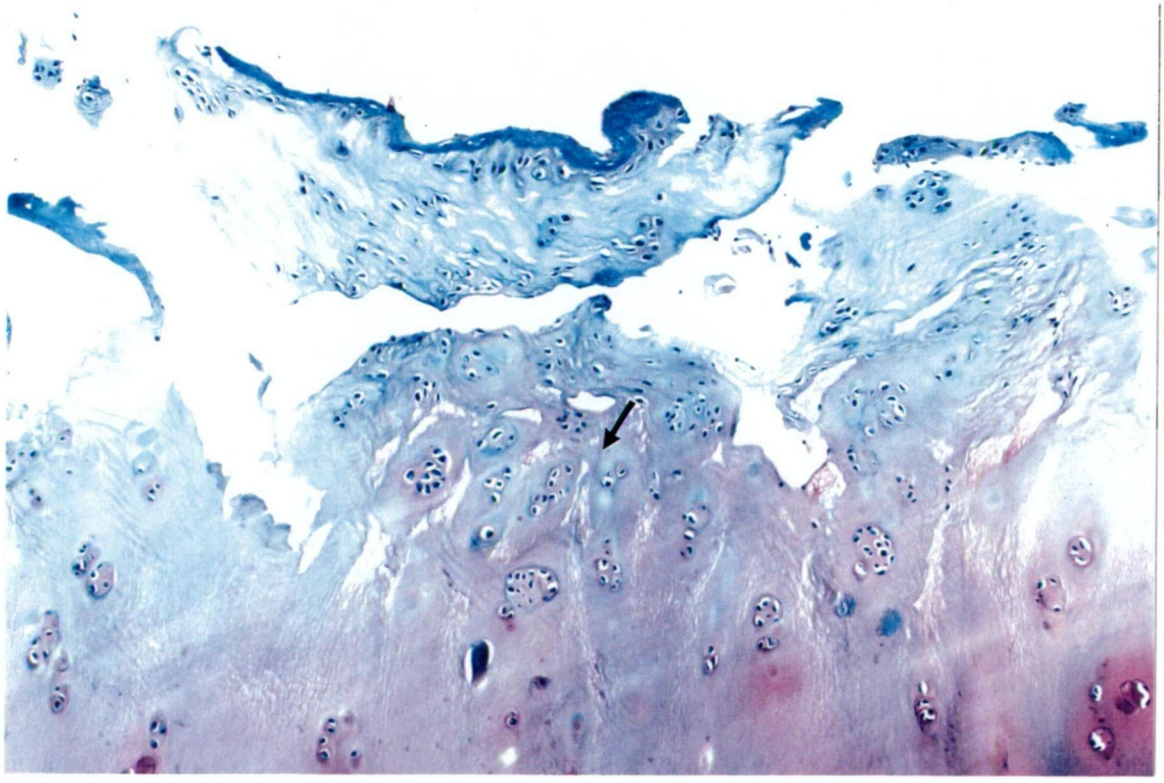


Figure 4-36 Severe OA Zone I-III (Toluidine Blue)

Pale TB staining with arrow pointing to area with multiple clones.

4.4.2 COLLAGEN STAINS

Picrosirius red staining indicated a normal cartilage distribution of collagen in normal groups i.e. with moderate staining of bone and calcified cartilage and increasing staining intensity towards the surface in uncalcified cartilage. This mirrors the ultrastructure where collagen fibrils arch superficially and compress to run parallel to the articular surface. This produces increased collagen density and is reflected by the stronger staining. It was also noted that there was a distinctive increase in the pericellular region immediately adjacent to the chondrocytes. The staining pattern became very disrupted in moderate and severe OA, with areas of pallor and of intense staining throughout the uncalcified cartilage matrix. Staining around the clones was bizarre often with intense staining of the matrix between and around these clusters of chondrocytes. The tidemark stained red whether single in normals or multiple in OA. As with the matrix in OA the staining of bone was very variable, some areas losing the uniform staining to show areas of both reduced and increased staining intensity.

A summary of these features is provided in Table 4-5.
Figure 4-30 and Figures 4-37 to 4-40 show examples of these features.

Table 4-5 Summary of Collagen Stain – Picrosirius Red

CARTILAGE GRADE	STAINING FEATURES
NORMAL	Surface - strong staining. Less staining with increasing depth Zone I>II>III>IV. Zone III, IV. pericellular stain. Chondrocytes - poor staining. Tidemark stains. Mild Zone V staining. Bone stains strongly.
AGED	Same as normal. Perhaps increased Zone III-IV pericellular stain.
EARLY OA	Similar to aged.
MILD OA	Decreased upper zone staining. Pale Zone III-IV stain. Pericellular pattern persists, less marked now. Clones stain pericellularly. Tidemark duplication. Mild decrease in subchondral plate stain.
SEVERE OA	Surface stain quite strong. More deep Zone IV staining. Pericellular pattern. Clones bizarre staining. Stronger staining with eburnated bone. Vascular structures stain more prominent.

Figure 4-37 Full Thickness Normal Cartilage (PSR)

Full thickness section showing strongest collagen staining in the superficial zones and bone in normal cartilage with Picrosirius Red.

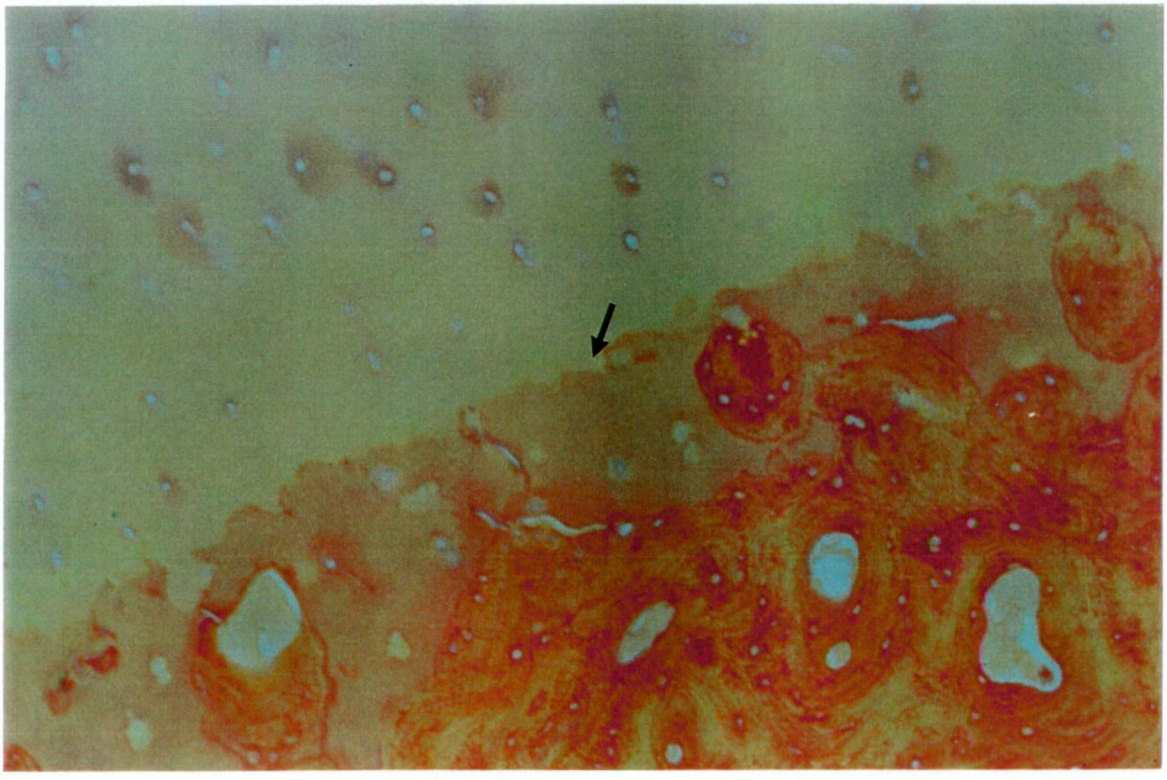


Figure 4-38 Chondro-Osseous Junction in Normal Cartilage (PSR)

Section of chondro-osseous junction demonstrating graduation of collagen staining bone >calcified cartilage > uncalcified cartilage. Arrow points to tidemark region.

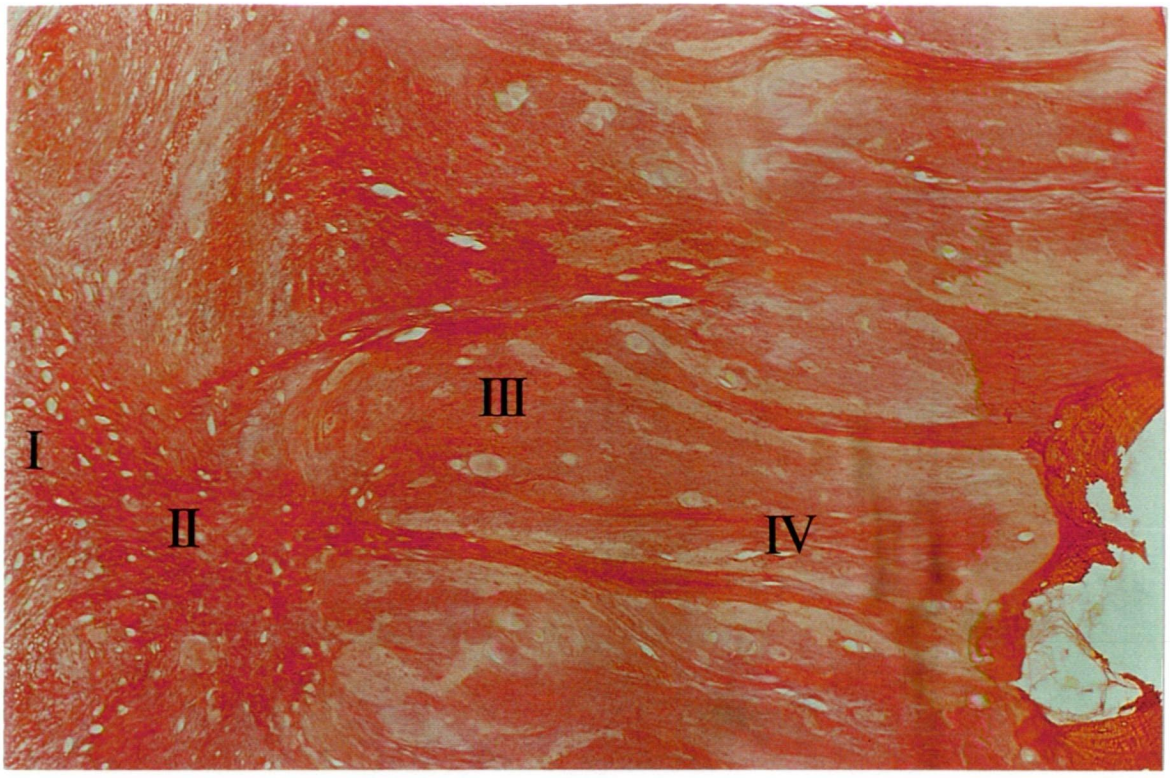


Figure 4-39 Full Thickness Normal Cartilage (PSR)

Some sections demonstrated the pattern of collagen fibres rising vertically through Zones III and IV and forming arcades in Zones I and II.

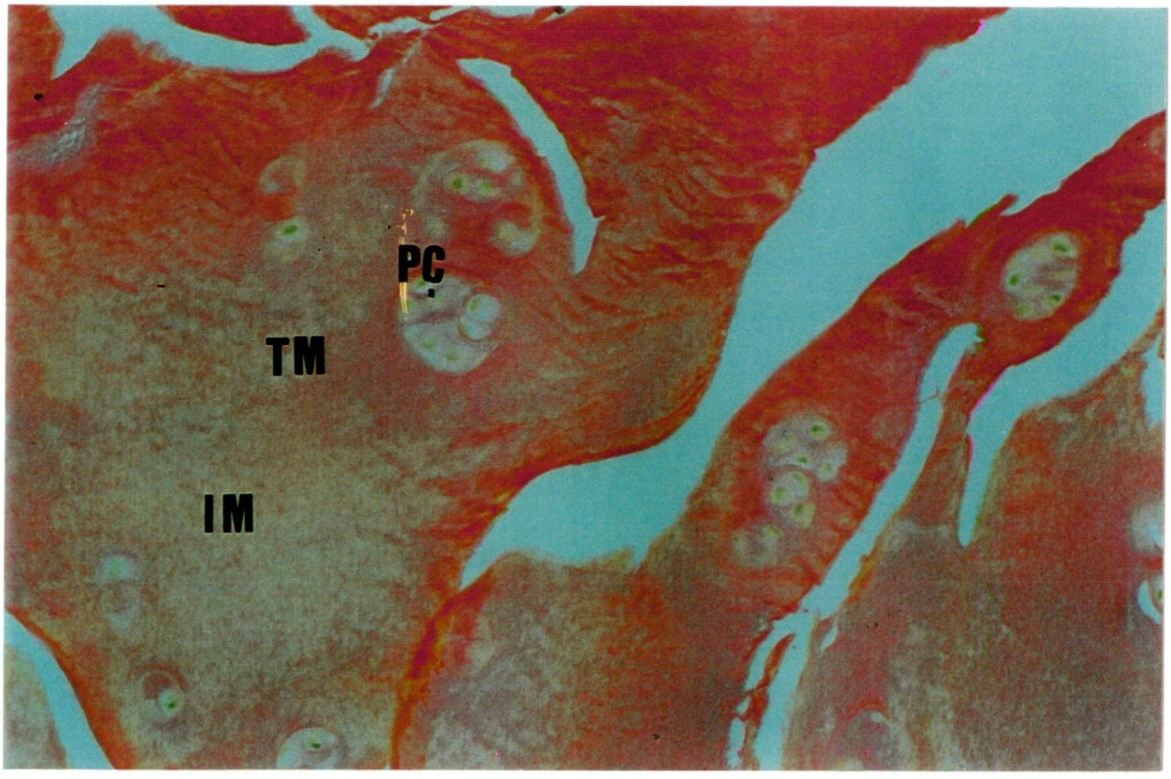


Figure 4-40 Fibrillated Cartilage in Severe OA (PSR)

Patchy matrical staining in fibrillations with relatively greater staining in pericellular (PC) region over territorial (TM) and interterritorial matrix (IM).

4.5 TIDEMARK: NORMAL MORPHOLOGY, HISTOCHEMISTRY AND 3D RECONSTRUCTION

The basophilic line seen on H & E staining at the junction of calcified and uncalcified cartilage in mature human articular cartilage represents the mineralisation front of the calcified cartilage. Both the microanatomical functions of the tidemark and its physiochemical properties remain very much an enigma. With proteoglycan stains the tidemark exhibited variable staining properties, with safranin O it was seen as a bright orange–red line, with toluidine blue as a deep navy blue line and with alcian blue, interestingly, there was no staining. With the collagen stain picrosirius red there was a strong crisp red line and adjacent to the calcified cartilage a zone of hazy yellowish staining. In normal joints interdigitations of uncalcified cartilage followed exactly by the tidemark dipped into the calcified cartilage and in places abutted onto subchondral bone and marrow spaces appearing to form a direct communication between the marrow spaces and uncalcified cartilage (Figs 4-41 to 4-46). Chondrocytes present in these prolongations appear on two dimensional sections to be entombed in calcified matrix. An initial simple physical 3D model of the tidemark constructed by photographing every 10th sequential slice over 100 sections of an area of clearly defined tidemark. Then, the chondro-osseous junction region was "cut out" and overlaid quite clearly demonstrating that uncalcified cartilage pegs run through the calcified cartilage into marrow spaces, the entombed chondrocytes being a 2-dimensional artefact (Fig 4-47). Using the computerised 3D reconstruction programme a 3D image of the tidemark was obtained. This programme captured grey images of the tidemark either directly from the microscope or from a black and white image, and converted the grey scales into a coloured image. In this case, 50 consecutive cases were fed into the system and this allowed reconstruction of one of the interdigitating uncalcified chondrocyte pegs. The peg was seen dipping through the calcified cartilage and enveloping an interdigitation of subchondral bone in which there was a marrow space (Figures 4-48 to 4-51).

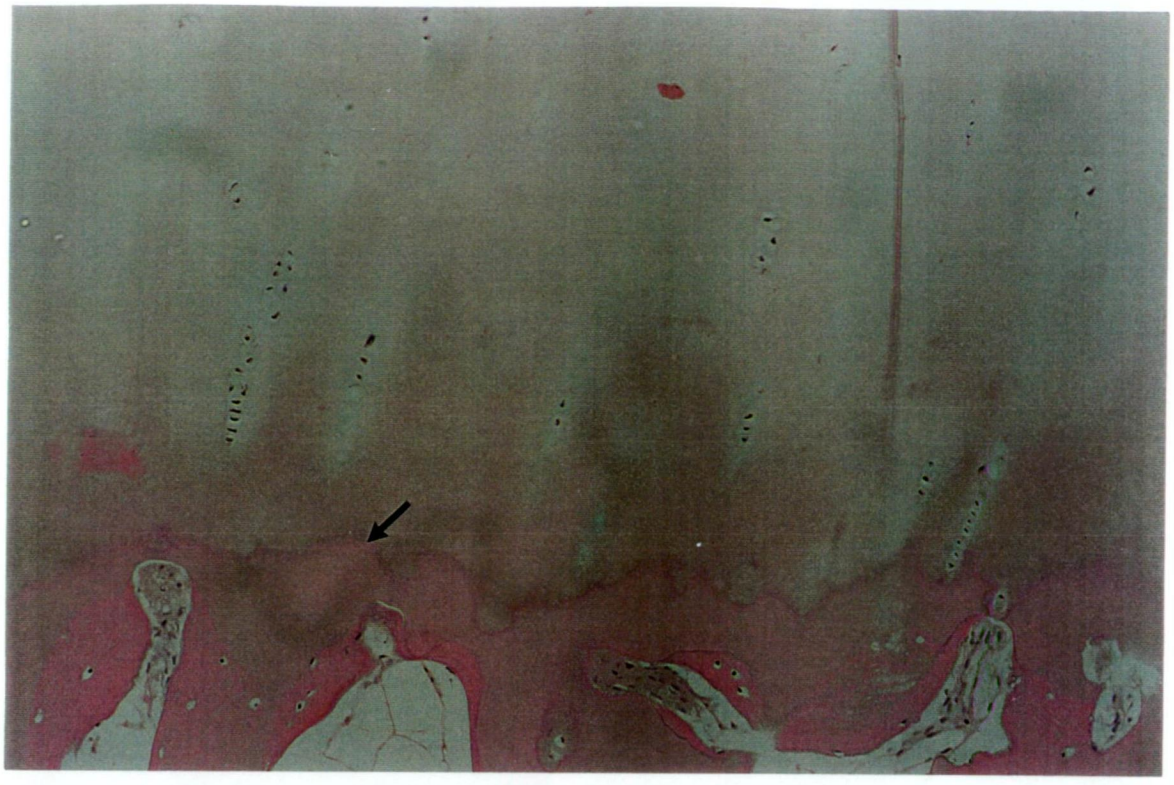


Figure 4-41 Tidemark Normal Cartilage (H & E)

Single tidemark stains purple with H & E stain. Arrow points to tidemark.

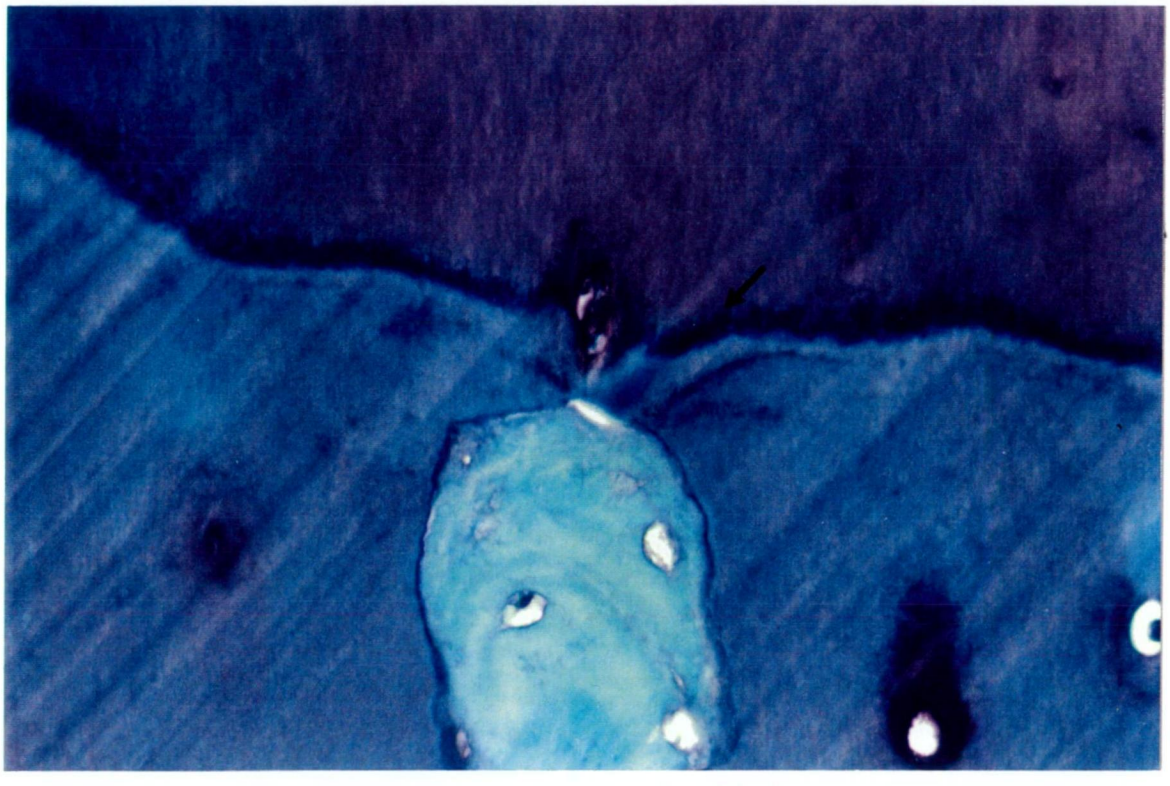


Figure 4-42 Tidemark Normal Cartilage (Toluidine Blue)

Single tidemarks stains deep blue with Toluidine Blue stain. Arrow points to tidemark.

Figure 4-43 Tidemark Normal Cartilage (SO)

Single tidemark stains yellow with Safranin O stain, demonstrated by the arrow.

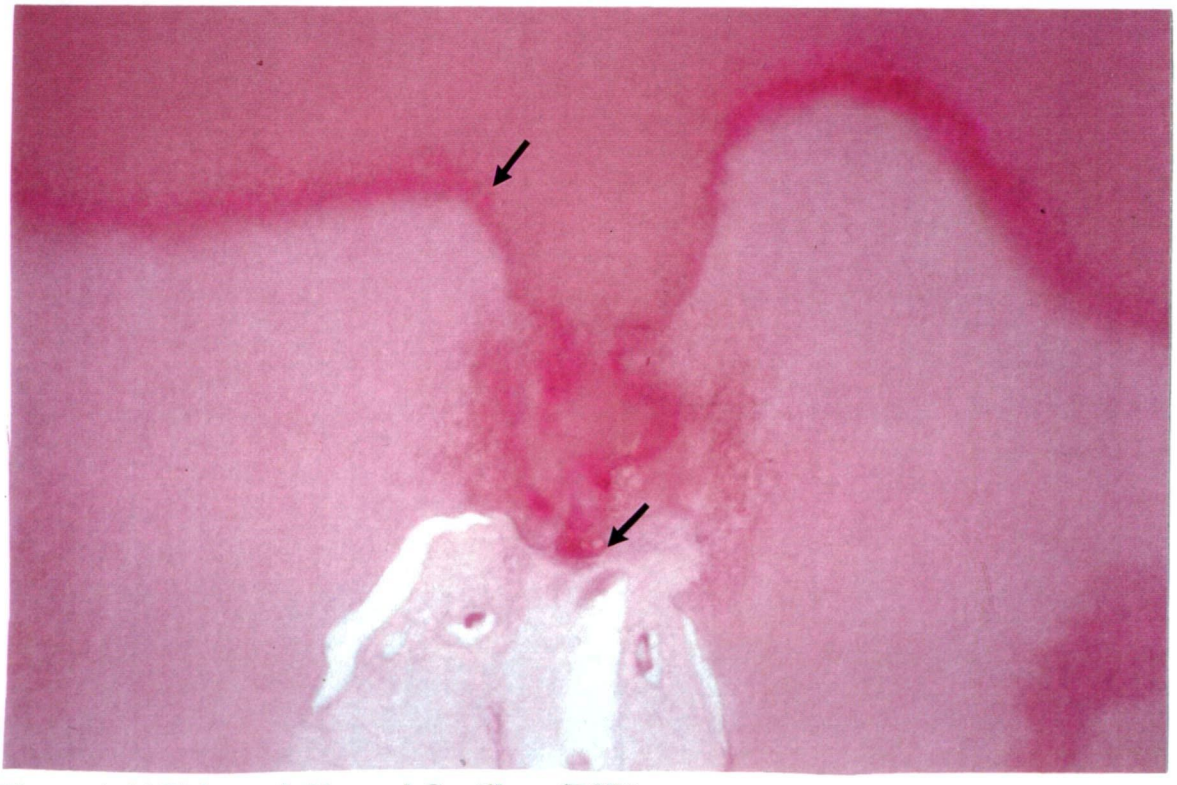


Figure 4-44 Tidemark Normal Cartilage (PSR)

Single tidemark stains red with PSR (upper arrow). Tidemark seen following uncalcified cartilage pegs dipping through calcified cartilage and abutting onto bone marrow spaces (lower arrow).

Figure 4-45 Uncalcified Cartilage Pegs (Toluidine Blue)

Pegs of uncalcified cartilage stained intensely with Toluidine Blue dip through the calcified cartilage and abut onto bone marrow spaces. Arrow points to this region.

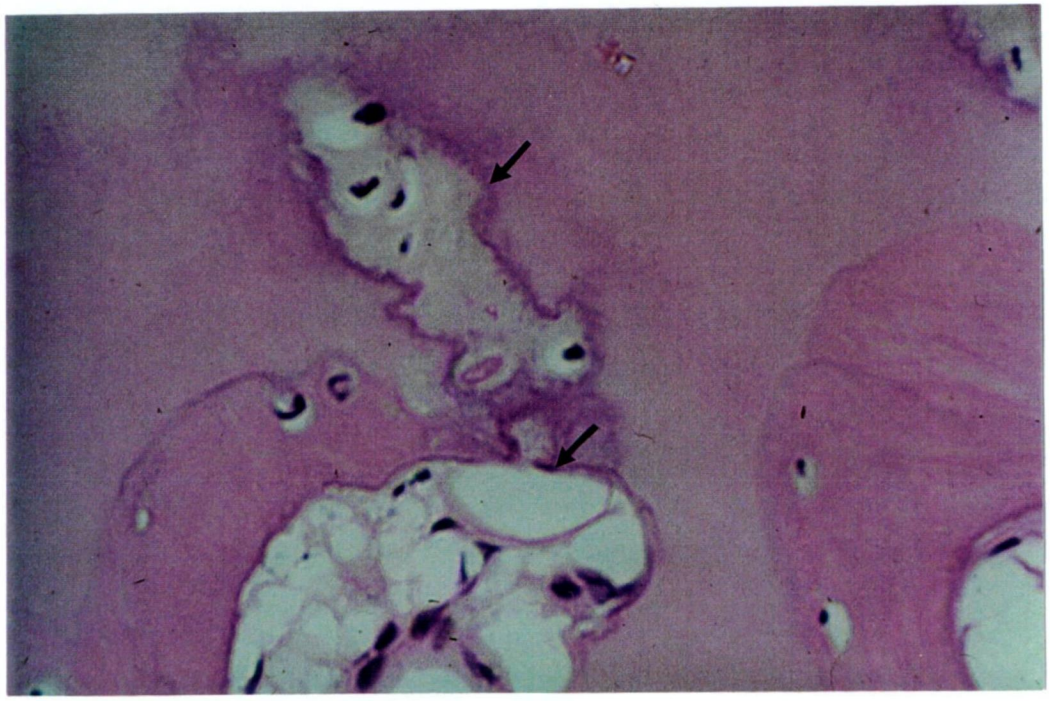


Figure 4-46 High Power View of Uncalcified Cartilage Peg (H & E)

Cartilage pegs faithfully followed by the tidemark dip through the calcified cartilage (upper arrow) and abut onto bone marrow spaces (lower arrow).

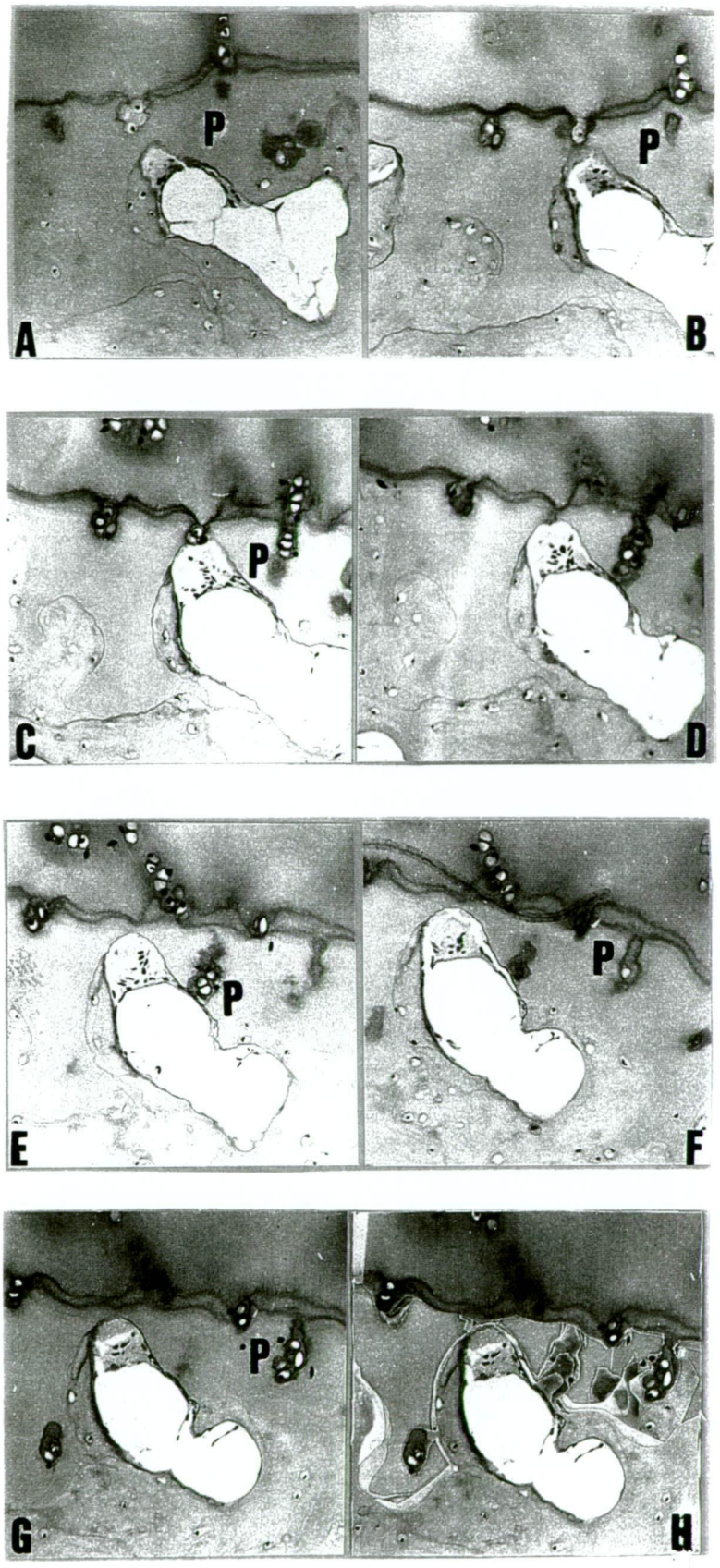


Figure 4-47 3D Reconstruction of Tidemark

Serial sections (A-G) from a normal tidemark region overlaid to show continuity of apparently discontinuous cartilage pegs(P) which abut onto a marrow space section(H). (Every tenth serial section was photographed in black and white and the pegs "cut out" and overlaid).

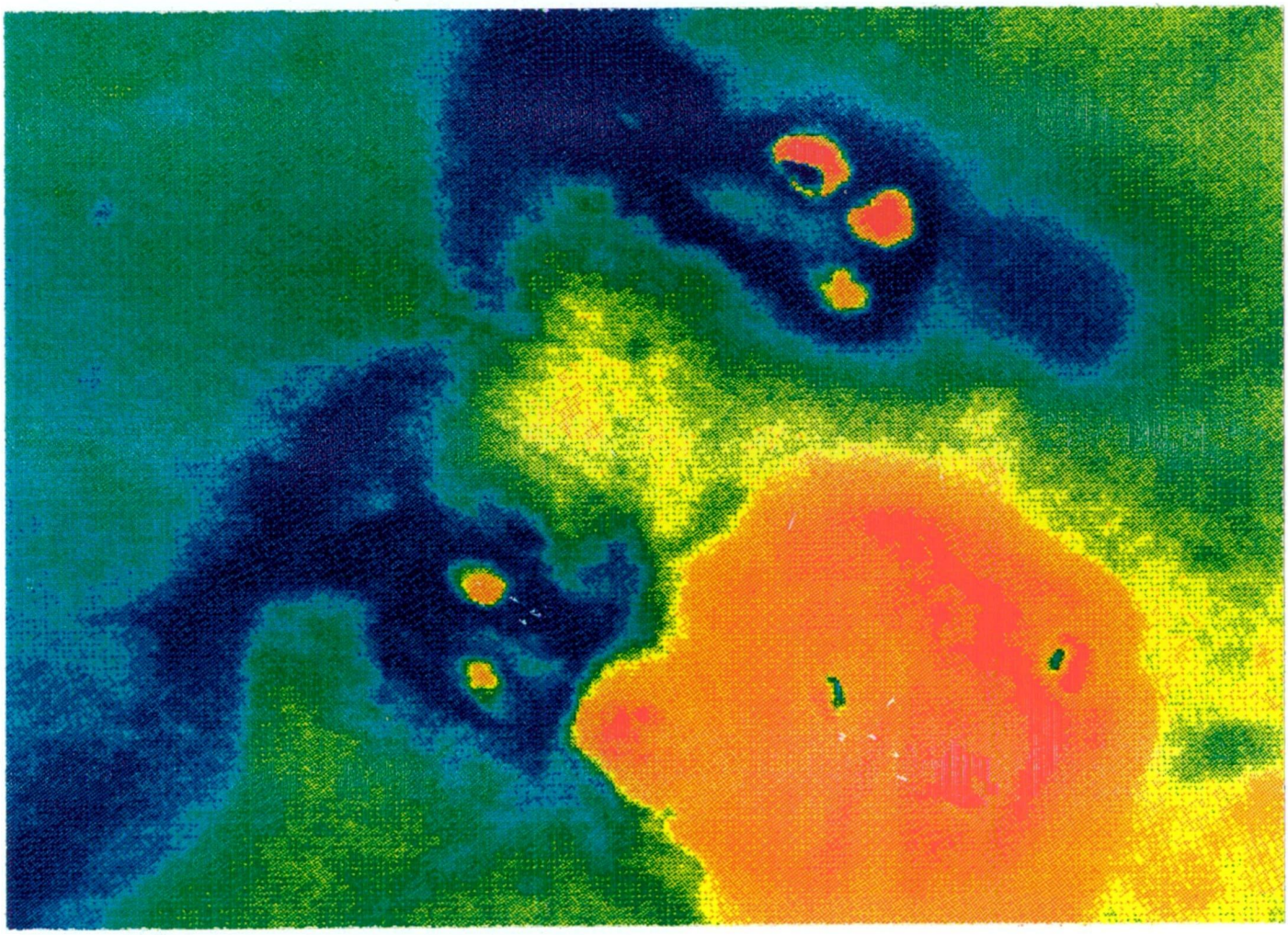


Figure 4-48 Coloured Computer Image of Grey Regions of Normal Tidemark

The computer programme interprets grey scales as different colours to allow overlaying of similar areas in 3D reconstruction programme. The tidemark is the blue line which is running across the left hand side of the picture. The green-blue areas represent uncalcified cartilage and the yellow-green areas calcified cartilage. The bone and marrow spaces are mainly red.

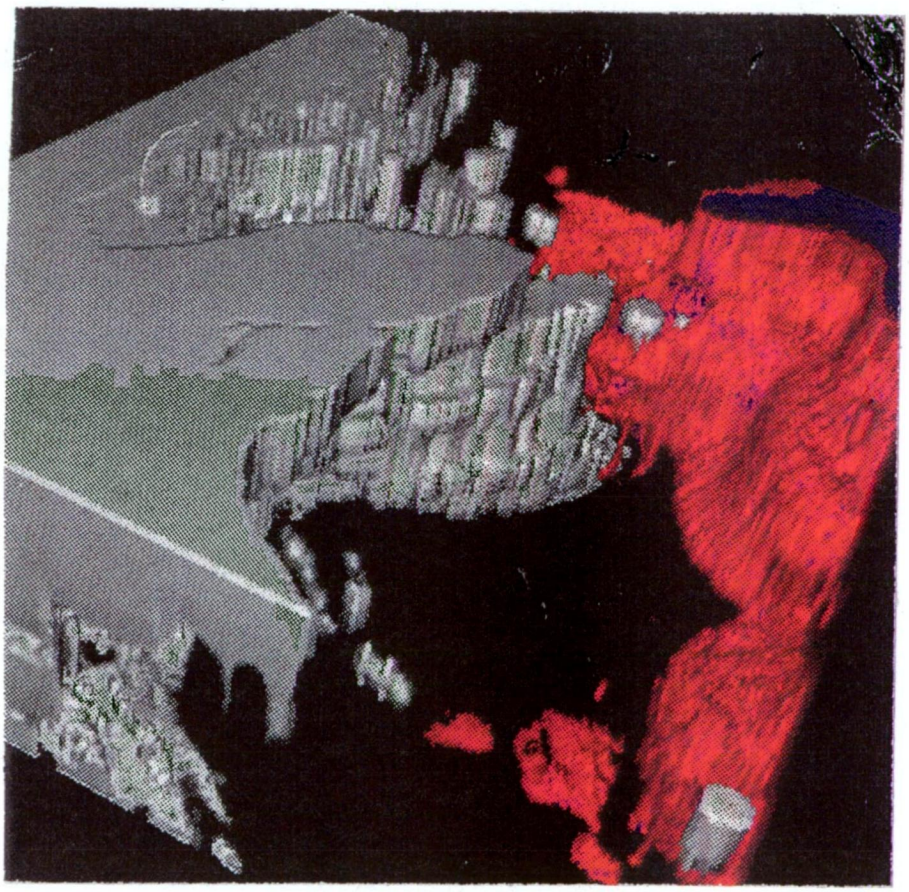


Figure 4-49 Computerised 3D Reconstruction of the Tidemark (Panoramic View)

In this image the grey regions represent subchondral bone and the red uncalcified cartilage pegs, the black in between is the calcified cartilage. The pegs are seen abutting and overlapping the subchondral bone.

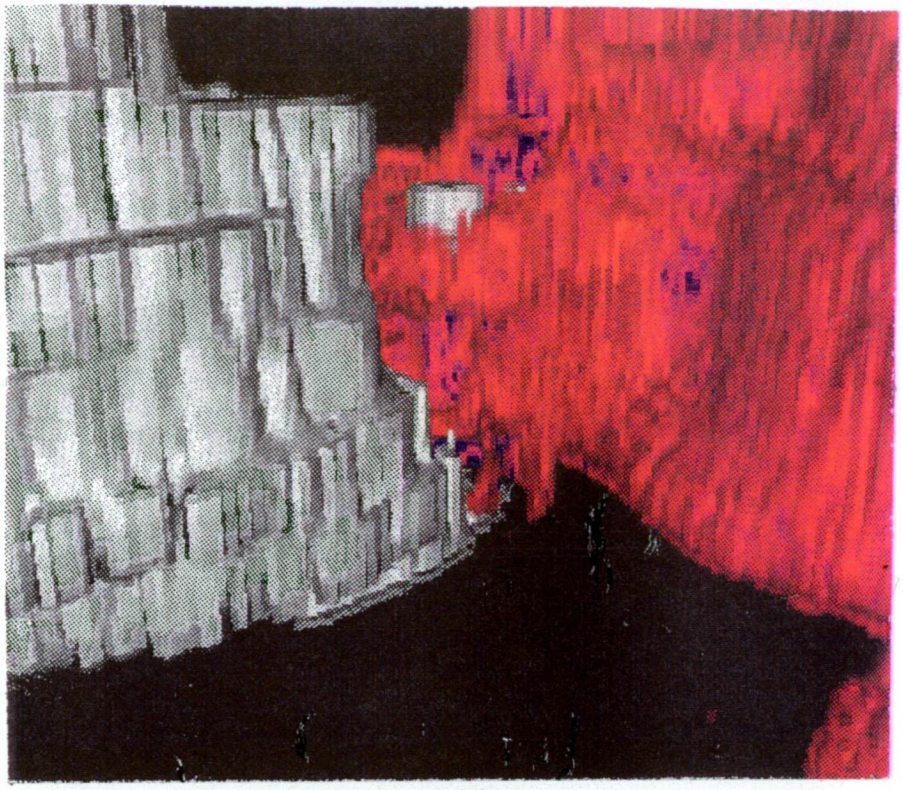


Figure 4-50 Lateral View of Image in 4.4.9

Uncalcified cartilage peg abutting onto subchondral bone interdigitation [bone (grey), calcified cartilage (black), peg (red)]

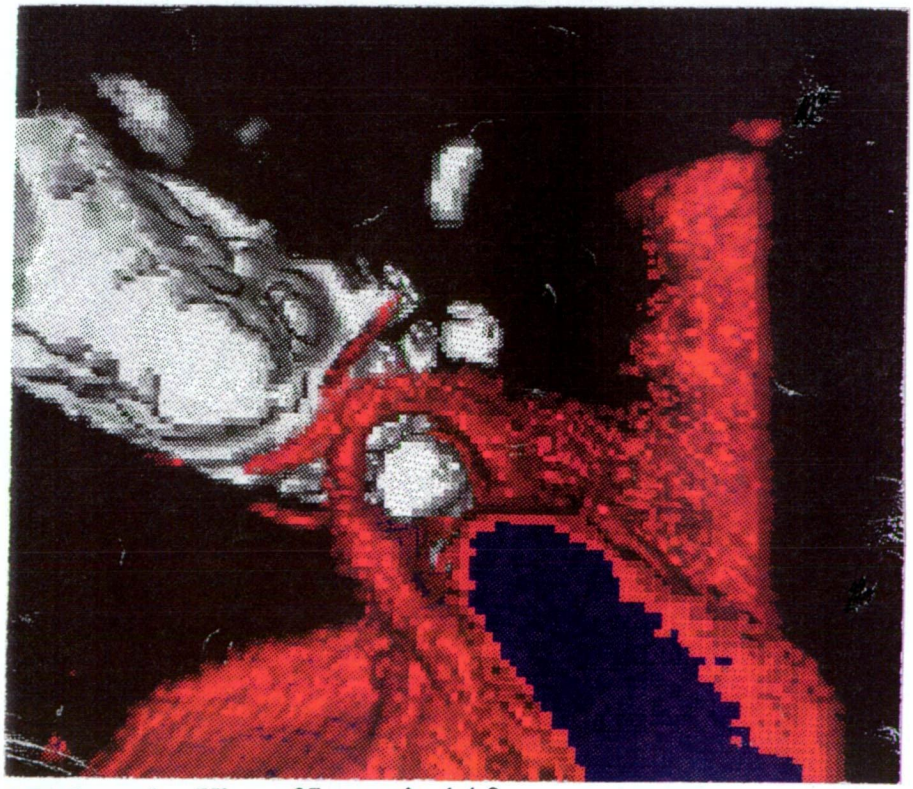


Figure 4-51 Superior View of Image in 4.4.9

Uncalcified cartilage peg enveloping subchondral bone interdigitation [bone (grey), calcified cartilage (black), peg (red)]

4.6 S-100 STAINING

S-100 staining was studied in the medial and lateral tibial plateaux of 31 cases comprised of 12 with unicompartmental OA (aged range 47-78), 11 with bicompartmental OA (age range 68-73) and 8 normals (age range 27-70). These cases represented the 31 cases from the main group. Of particular interest where the changes seen in early osteoarthrosis, which was characterised by: chondrocyte proliferation and clone formation, decreased proteoglycan and profound disruption of the chondro-osseous junction.

In normals, there was prominent staining of chondrocyte cytoplasm and membrane in all zones. Pericellular and territorial matrix staining was seen in all zones but this was strongest in zones II and III. Staining of the interterritorial matrix was present but was weak in all zones. Tidemark staining was negative in all sections examined. In overt OA and early OA there was a disruption of this typical staining pattern with variable matrical staining and a characteristic increase in and around chondrocyte clones. The lack of staining at the tidemark in the chondro-osseous region was surprising since S100 is a known calcium ion transport molecule. One would expect there to be disturbed calcification mechanisms in zone V, however, if this was the case these changes are not demonstratable using this S100 protein staining technique. Figures 4-52 to 4-57 show the key features.

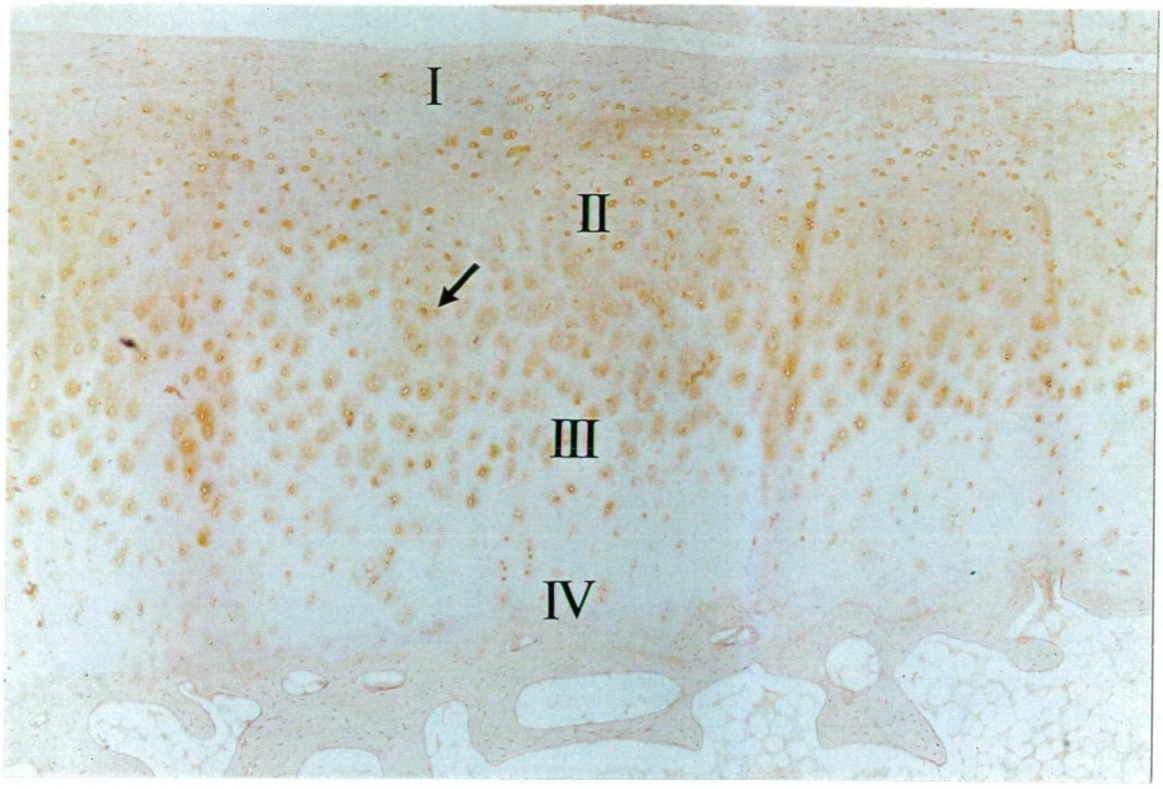


Figure 4-52 S-100 Protein Staining Full Thickness Normal Case

Moderate staining of chondrocyte cytoplasm and membrane and pericellular matrix (arrow) and patchy mild territorial matrix staining in normal group with S-100 protein.

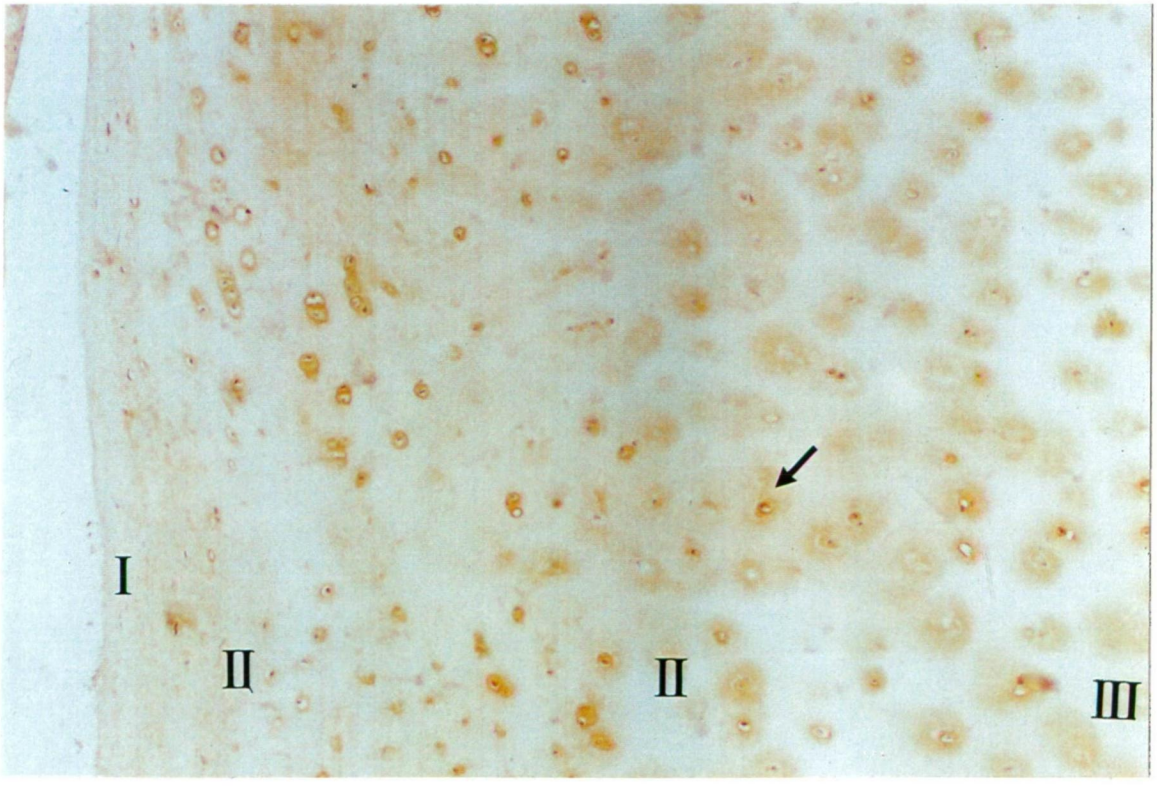


Figure 4-53 S-100 Protein Staining Zone I-III Normal Case

Zones I-III show chondrocyte cytoplasm and membrane staining. There is also pericellular (arrow) and territorial matrix staining greater in zones I and II.

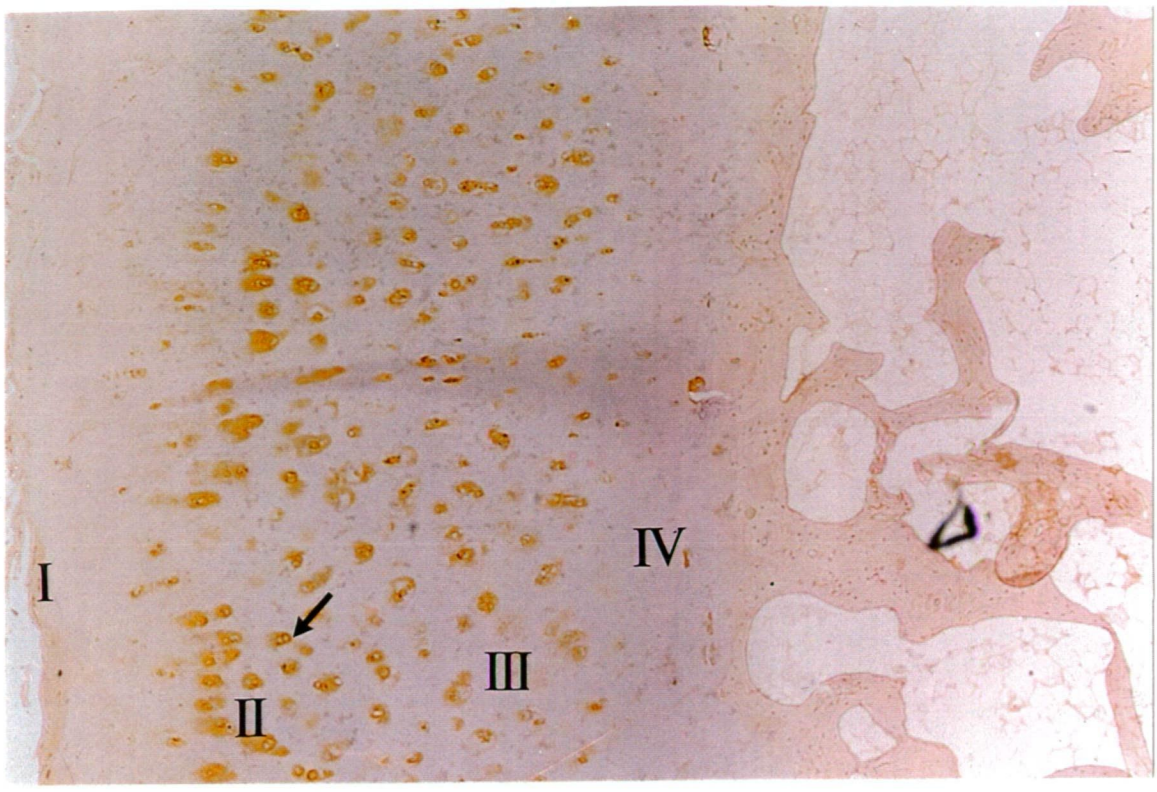


Figure 4-54 S-100 Protein Staining in Early OA

Relative to normal there is increased chondrocyte cytoplasm, membrane and pericellular staining. The arrow points to chondrocytes.

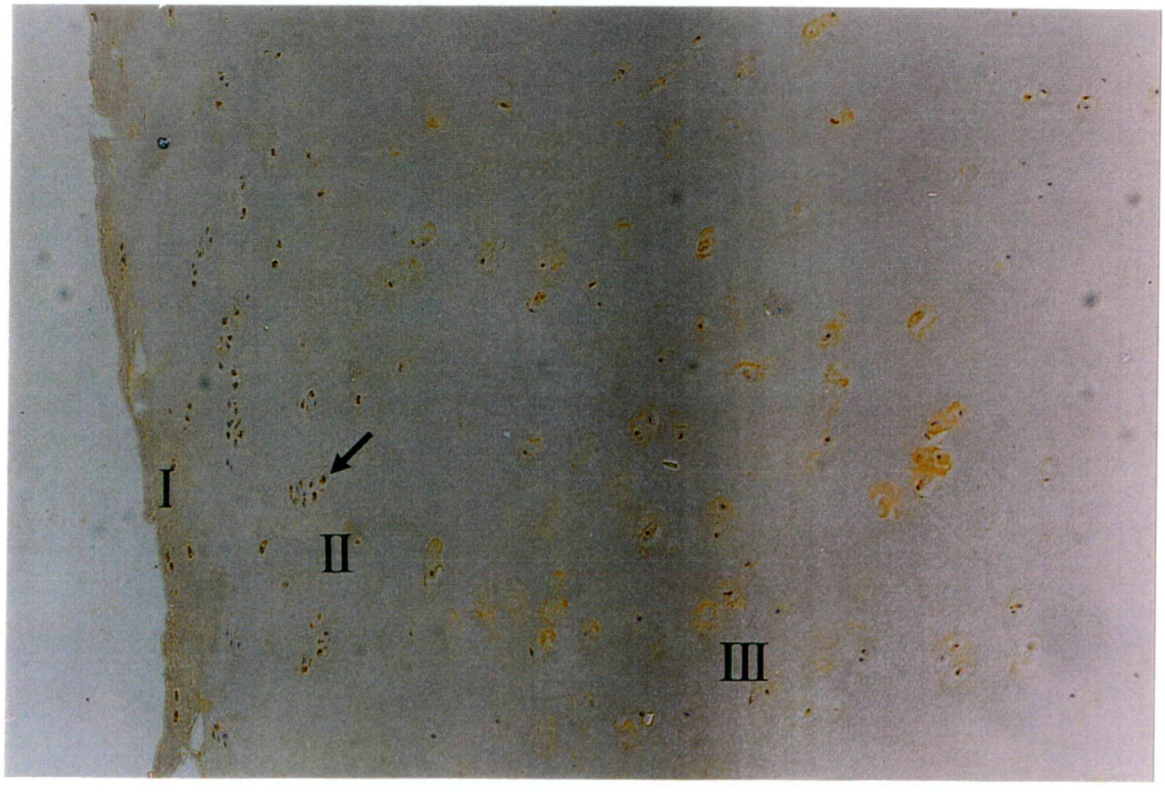


Figure 4-55 S-100 Protein Staining of Clones in Early OA in Zone I-III

Where present small clones stain in early OA. Arrow points to a small clone.

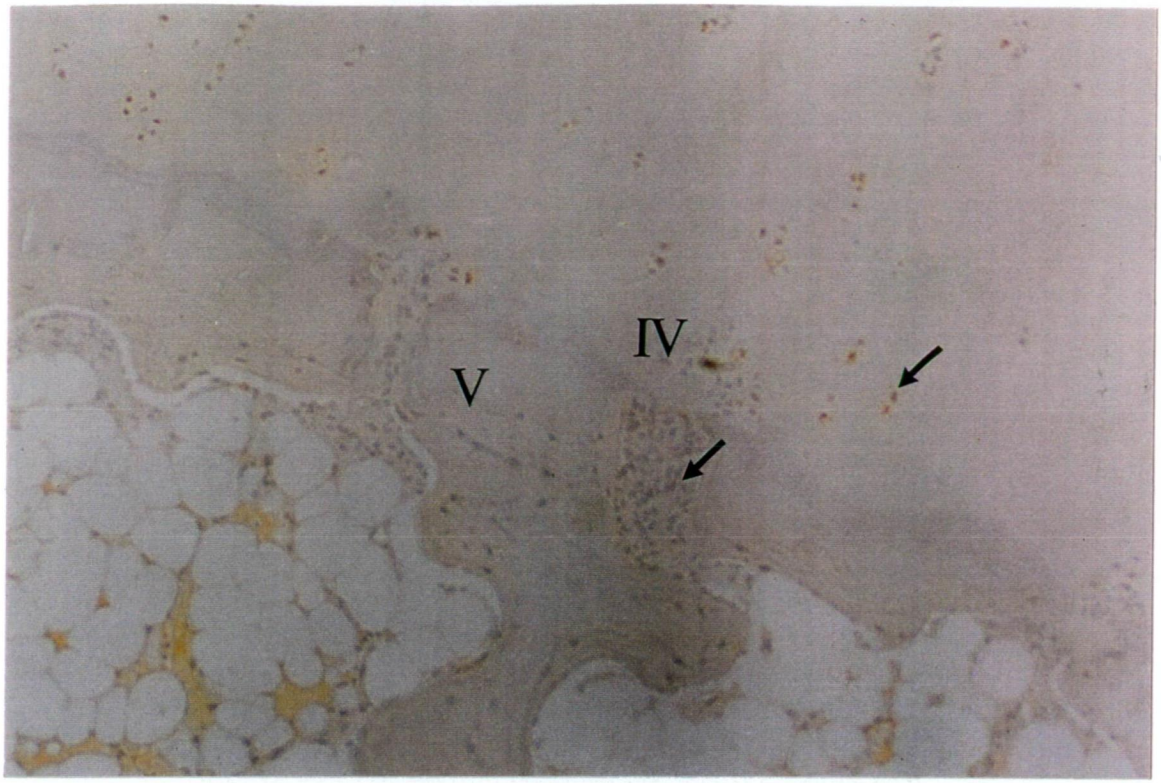


Figure 4-56 S-100 Protein Staining at Chondro-Osseous Junction in Early OA

Chondrocyte clone staining in zone IV and patchy staining of vessels that are extending through tidemark (lower arrow).

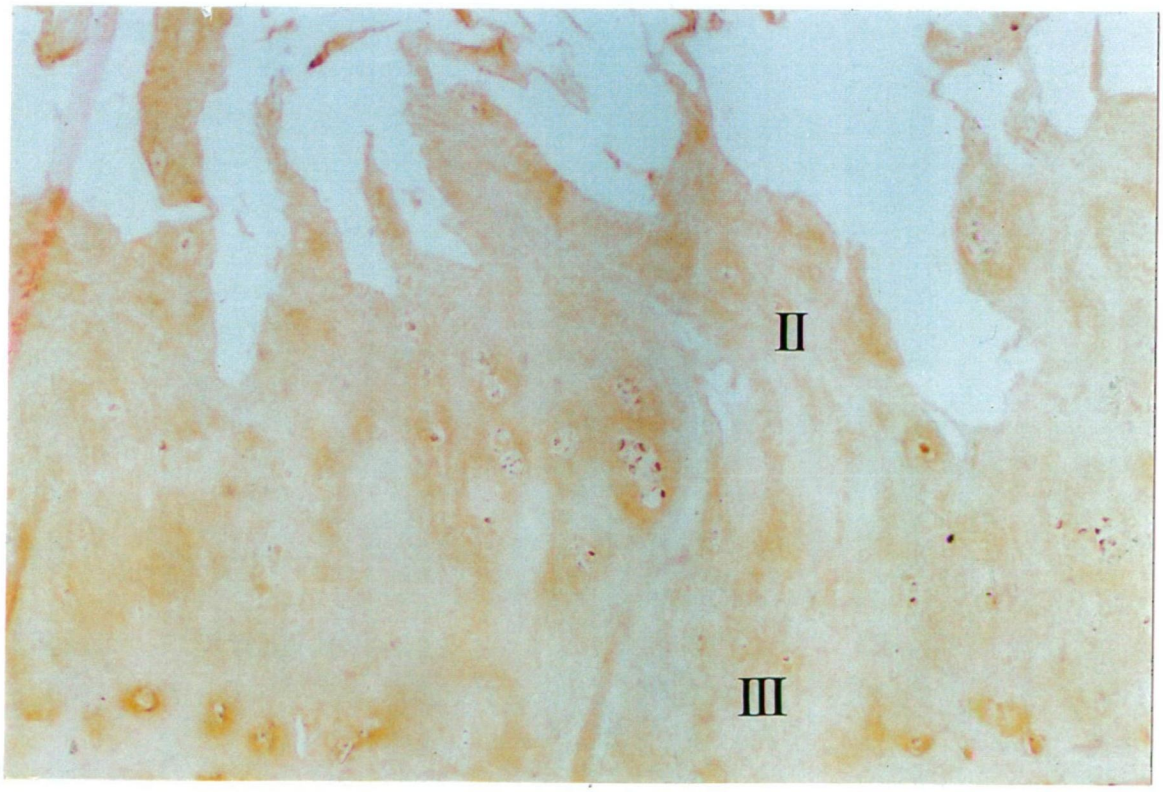


Figure 4-57 S-100 Protein Staining in Severe OA

Fibrillated cartilage and zones II and III staining, patchy staining of clones and matrix.

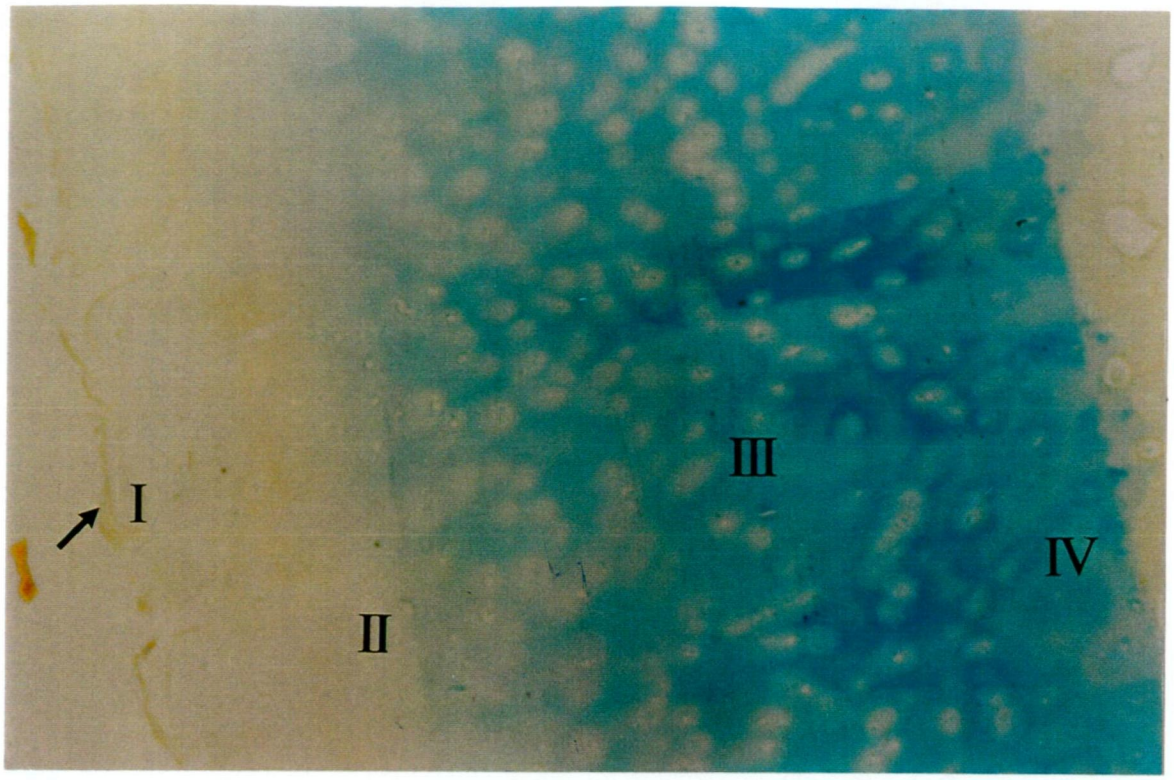


Figure 4-58 GNA Lectin Staining in Ageing Cartilage

There is very mild staining of the surface (arrow) and matrix in the upper zones.

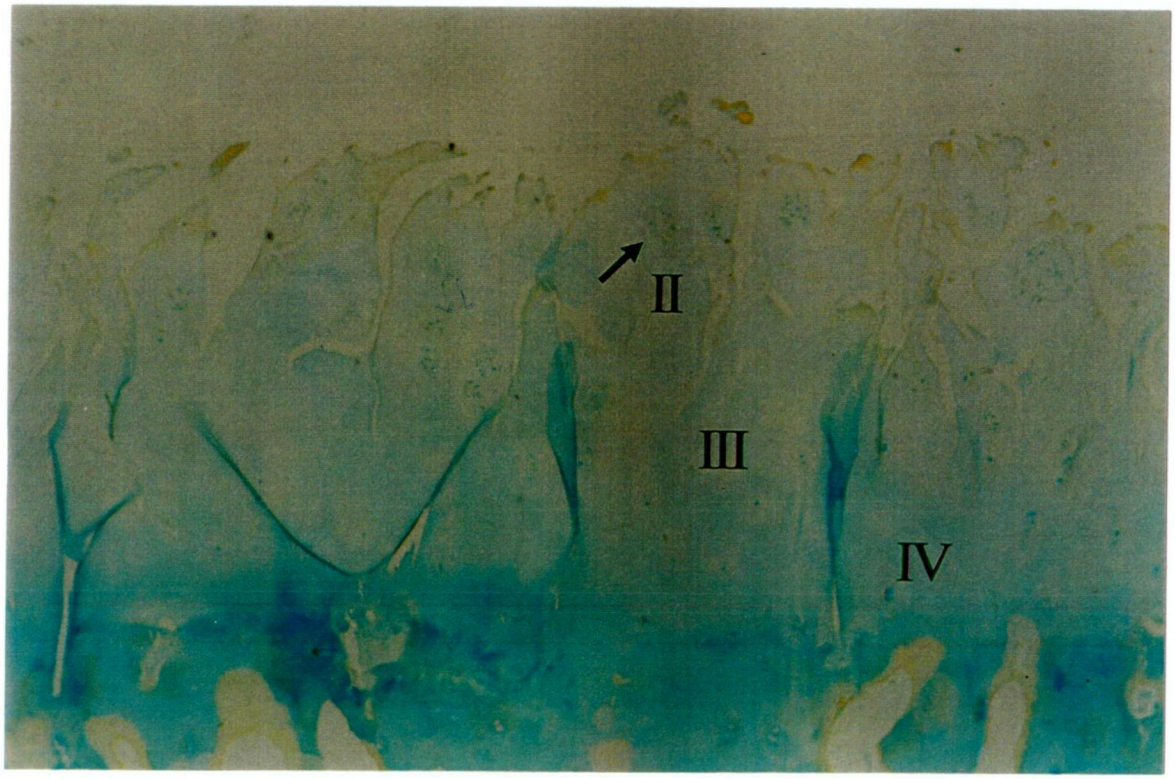


Figure 4-59 GNA Lectin Staining in Severe OA

There is very weak surface staining; this is absent in chondrocyte and matrix (arrow).

4.7 LECTIN STUDIES

The lectins can be assigned into groups according to their specific glycosidic residue binding characteristics. Because some lectins are multivalent in this respect the results can be discussed in terms of lectin groups as well as considering some individual lectins that have dominant singular binding specificities. Results are presented as a discussion of the semi-quantitative analysis of the binding affinities of the battery of lectins. There is a summary table of the key binding affinities with appropriate photographs of the binding characteristics. The results from the ***preliminary trial*** of 29 lectins are summarised below:

Group 1 - α - mannose : GNA=*NPA* in staining characteristics.

Group 2 – complex N linked: *ConA* – stains virtually all cells and matrix, LCA – similar pattern to ConA but stronger intensity of staining, PSA – predominantly cells, EPHA – similar pattern to ConA/LCA, with greater surface staining, LPHA – surface predominantly.

Group 3 – N-acetyl lactosamine, di-N-acetylchitobiose: DSA = LEA – all chondroctyes and matrix, STA – very variable pattern in chondroctyes and matrix, *PWM* – nothing, WGA – broad based staining, BSA – II – nothing.

Group 4 - β - galactose: MPA – good broad based staining, *AHA* – very little staining, ECA – occasional chondrocytes, little matrix, CTA – surface and chondrocytes.

Group 5 – N-acetyl galactosamine: DBA – broad based staining matrix and cells, SBA – nothing, VVA B4 – occasional cells, mild matrix, HPA = AHA – surface and chondrocytes, matrix to a lesser degree.

Group 6 – fucose: UEA – very weak staining, LTA – good surface, bone and chondrocytes.

Group 7 – sialic acid: MAA – predominantly chondrocytes, SNA – chondrocytes and superficial matrix, LFA – chondrocytes, matrix but not bone.

Group 8 - α galactose: *BSA B4* – nothing.

In summary, the final panel of lectins was: Group 1 – GNA, Group 2 – LCA, PSA, EPHA, LPHA, Group 3 – DSA, STA, WGA, Group 4 – MPA, ECA, CTA, Group 5 – DBA, VVA B4, WFA, HPA, Group 6 – LTA, Group 7 – MAA, SNA, LFA.

4.7.1 GROUP 1 - α MANNOSE

This group is represented by GNA that binds to mannose α 1,3 mannose.

4.7.1.1 GNA

SURFACE: very mild surface staining was present in the normals, aged and early OA group. This was absent in the mild OA group.

CHONDROCYTES: very mild patchy cytoplasmic staining was present in a small percentage of cells in less than 50% of the cases in the normals, aged and early OA groups. This was absent in the moderate and severe OA groups. No staining of chondrocyte cell membranes peg chondrocytes or clones was seen.

MATRIX: very mild zone I staining was present in the ageing and early OA groups, to a lesser extent in the mild OA group and was absent in normal and severe OA groups. Very mild zone II staining was seen in all regions in less than 50% of the cases in the ageing, early and mild OA groups with none in the normals or severe OA groups. There was no staining of zones III, IV and V cartilage pegs and clones.

CHONDRO-OSSEOUS JUNCTION: No staining of the tidemark, vessels, vessel matrix or splits.

SUBCHONDRAL BONE: No staining of osteocytes or bone matrix.

NEW CARTILAGE: No staining.

A summary of the key findings is provided in Table 4-6.

Examples of the staining patterns are seen in Figures 4-58 and 4-59.

Note: For each table staining was scored 0=absent, 1=mild, 2=moderate, 3=strong, 4=very strong.

Chondrocyte cytoplasm (CY), chondrocyte membrane (CM), pericellular (PC), interterritorial (IM), territorial (TM).

Table 4-6 Summary of GNA lectin histochemistry – Group 1: α Mannose

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	1	1	1	1	1/1	0/0	0	0/0	0/0	0	0/0	0/0	0	0	0	0
1 EARLY	1	1	1	1	1/1	1/1	1	0/0	0/0	0	0/0	0/0	0	0	0	0
2 AGED	1	1	1	1	1/1	1/1	1	0/0	0/0	0	0/0	0/0	0	0	0	0
3 MOD	0	0	0	1	0/0	1/1	1	0/0	0/0	0	0/0	0/0	0	0	0	0
4 SEVER	0	0	0	1	0/0	0/0	0	0/0	0/0	0	0/0	00	0	0	0	0

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was mild CY and CM in zones I and III in the normals, ageing and early OA groups.
Matrix: weak staining was seen in all groups in zone I, in the middle three groups in zone II and was otherwise absent.
Tidemark: no staining. Osteocytes and bone matrix: no staining.

4.7.2 GROUP 2 – COMPLEX N-LINKED

This group is represented by lectins which recognise complex N-linked sequences and includes LCA, PSA, EPHA and LPHA.

4.7.2.1 LCA

This lectin stains non-bisected bi/tri antennary sequences.

SURFACE: Moderate staining in the normal, ageing and OA groups.

CHONDROCYTES: The chondrocyte cytoplasm and membrane stained moderately in all cells and zones in the normals. Cytoplasm and membrane staining remained moderate in the ageing and OA groups, however, there was a slight decrease in the overall percentage of cells staining. The cytoplasm of the peg chondrocytes exhibited moderate staining in all groups, the percentage of cells staining was between 40-60% in the normal group and in the other groups was variable being between 0-60%. In these peg cells membrane staining was present in 40-60% of cases in the normal, ageing and early OA groups and was absent in the more severe OA groups. There was moderate to strong cytoplasmic and membrane staining in the majority of clones in the ageing and OA groups.

MATRIX: staining in zones I and II in all regions and groups was similar with a mild to moderate staining intensity. In zones III and IV in the normal, ageing and early OA groups there was mild pericellular staining with a gradual decrease across the territorial and interterritorial regions with a more pronounced difference in zone IV. In the mild and severe OA groups staining was very weak in all regions in zones III and IV. There was no staining in zone V or peg matrix. There was mild to moderate staining of the intra- and periclonal matrix.

CHONDRO-OSSEOUS JUNCTION: tidemark and vessel staining was absent and that of the vessel matrix and splits was mild-moderate in all groups.

SUB-CHONDRAL BONE: osteocytes and bone matrix show mild staining.

NEW CARTILAGE: none of the parameters for new cartilage stained.

A summary of the key findings is provided in Table 4-7.

Examples of the staining pattern are seen in Figures 4-60 and 4-61.

Table 4-7 Summary of LCA lectin hisochemistry – Group 2: Complex N-Linked

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	2	2	2	2/2	2/2	2	2/2	2/1	0	2/2	2/1	0	0	2	2
1 EARLY	2	2	2	2	2/2	2/2	2	2/2	2/1	0	2/2	2/1	0	0	2	2
2 AGED	2	2	2	2	2/2	2/2	2	2/2	2/1	0	2/2	2/1	0	0	2	2
3 MOD	2	2	2	2	2/2	2/2	2	2/2	2/1	0	2/2	2/1	0	0	2	2
4 SEVER	2	2	2	2	2/2	2/2	2	2/2	2/1	0	2/2	2/1	0	0	2	2

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was moderate staining of CY and CM in all groups and zones.
Matrix: was moderate in zone I and II and decreased PC>>TM>IM in zones III and IV.
Tidemark: no staining. Osteocyte and bone matrix: moderate staining.

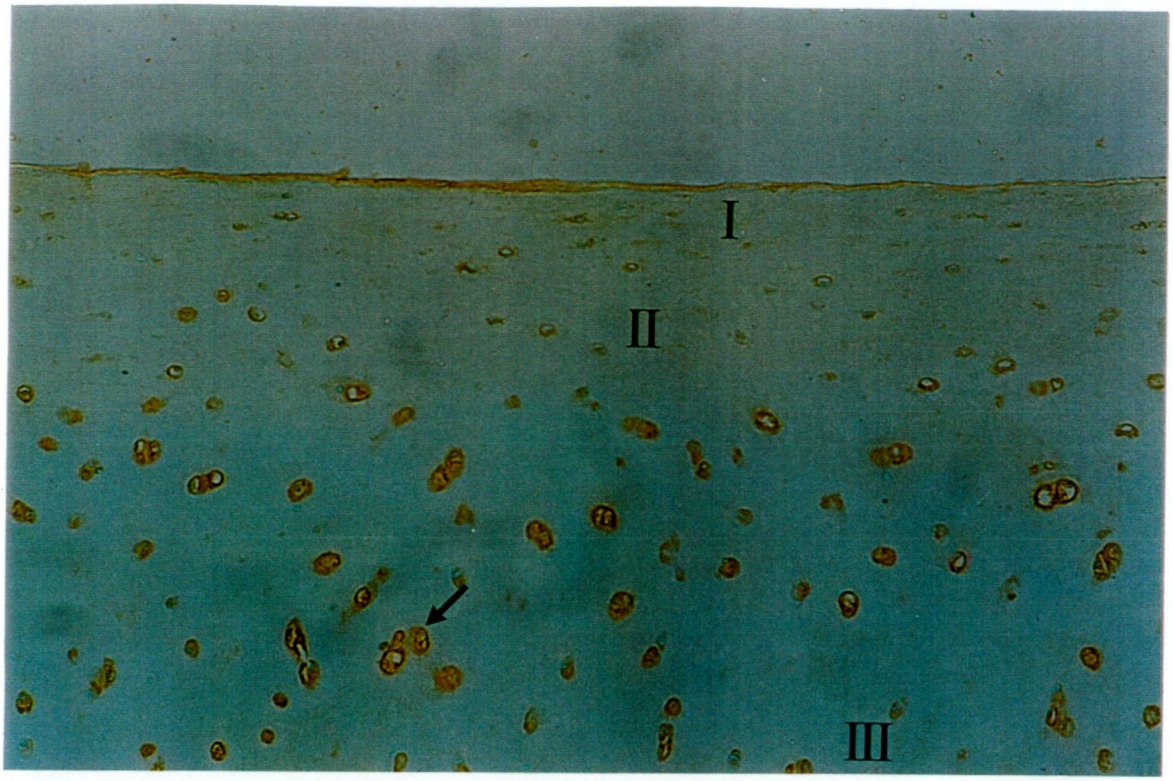


Figure 4-60 LCA Lectin Staining Zones I and II in Normal Cartilage

There is staining of cell membrane, cytoplasm and matrix. Arrow points to chondrocytes.

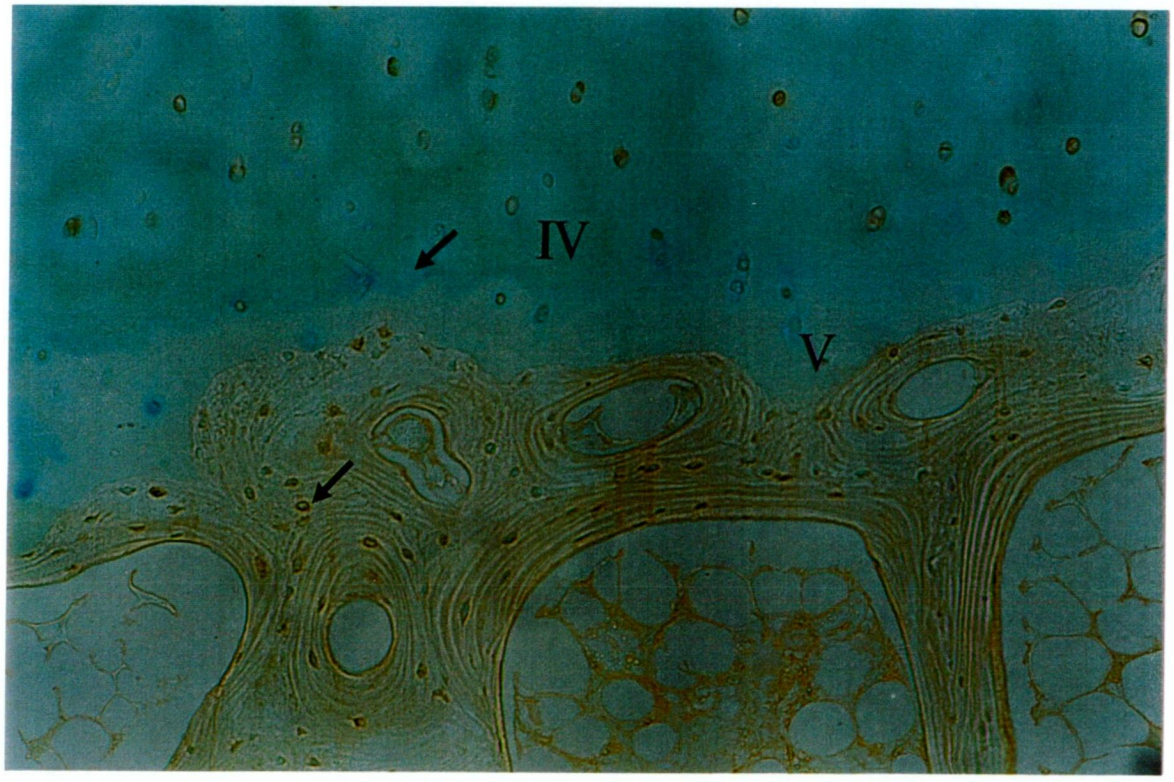


Figure 4-61 LCA Lectin Staining at Chondro-Osseous Junction in Normal Cartilage

In the tidemark staining is absent (upper arrow) and in chondrocytes and bone matrix (lower arrow) it is moderate.

4.7.2.2 PSA

This lectin stains high mannose, short chained and non-bisected sequences. Staining in all five groups was similar.

SURFACE: there was moderate to strong surface staining.

CHONDROCYTES: there was moderate to strong cytoplasmic and membrane staining in the majority of chondrocytes in all zones. The chondrocytes in the pegs showed a similar staining intensity but with a lesser percentage of cells staining. There was mild to strong clone staining in all cells in the ageing and OA groups.

MATRIX: there was mild to moderate matrical staining throughout the zones, with PC>TM>IM. There was weak patchy staining of zone V and pegs. Intraclonal and periclonal matrix showed mild to moderate staining.

CHONDRO-OSSEOUS JUNCTION: the tidemark and vessels did not stain. The vessel matrix and tidemark splits showed moderate to strong staining.

SUBCHONDRAL BONE: there was strong staining of osteocytes and bone matrix.

NEW CARTILAGE: where new cartilage was present the cytoplasm and membranes stained strongly and the matrix moderately.

A summary of the key findings in provided in Table 4-8.

Examples of the staining pattern are seen in Figures 4-62 and 4-63.

Table 4-8 Summary of PSA lectin histochemistry – Group 2: Complex N-Linked

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	3	3	3	3	3/3	3/2	1	3/3	3/2	1	3/3	3/2	1	0	3	3
1 EARLY	3	3	3	3	3/3	3/2	1	3/3	3/2	1	3/3	3/2	1	0	3	3
2 AGED	3	3	3	3	3/3	3/2	1	3/3	3/2	1	3/3	3/2	1	0	3	3
3 MOD	3	3	3	3	3/3	3/2	1	3/3	3/2	1	3/3	3/2	1	0	3	3
4 SEVER	3	3	3	3	3/3	3/2	1	3/3	3/2	1	3/3	3/2	1	0	3	3

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was moderate to strong staining of all CM and CY in all groups and zones.
Matrix: showed strong staining in groups I and II and decreased PC>TM>IM in zones III and IV.
Tidemark: no staining. Osteocytes and bone matrix: strong staining.

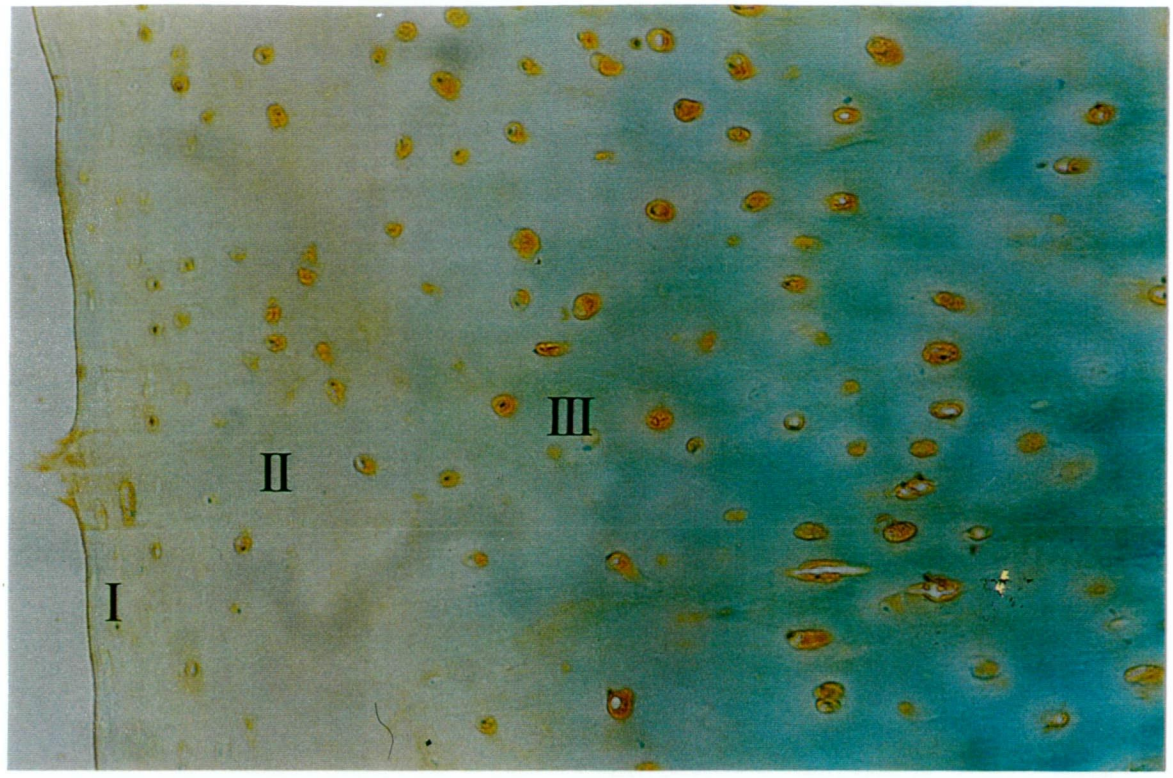


Figure 4-62 PSA Staining of Zones I-III in Early OA

The pattern is: surface moderate, cytoplasm and membranes moderate and matrix PC>TM>IM.

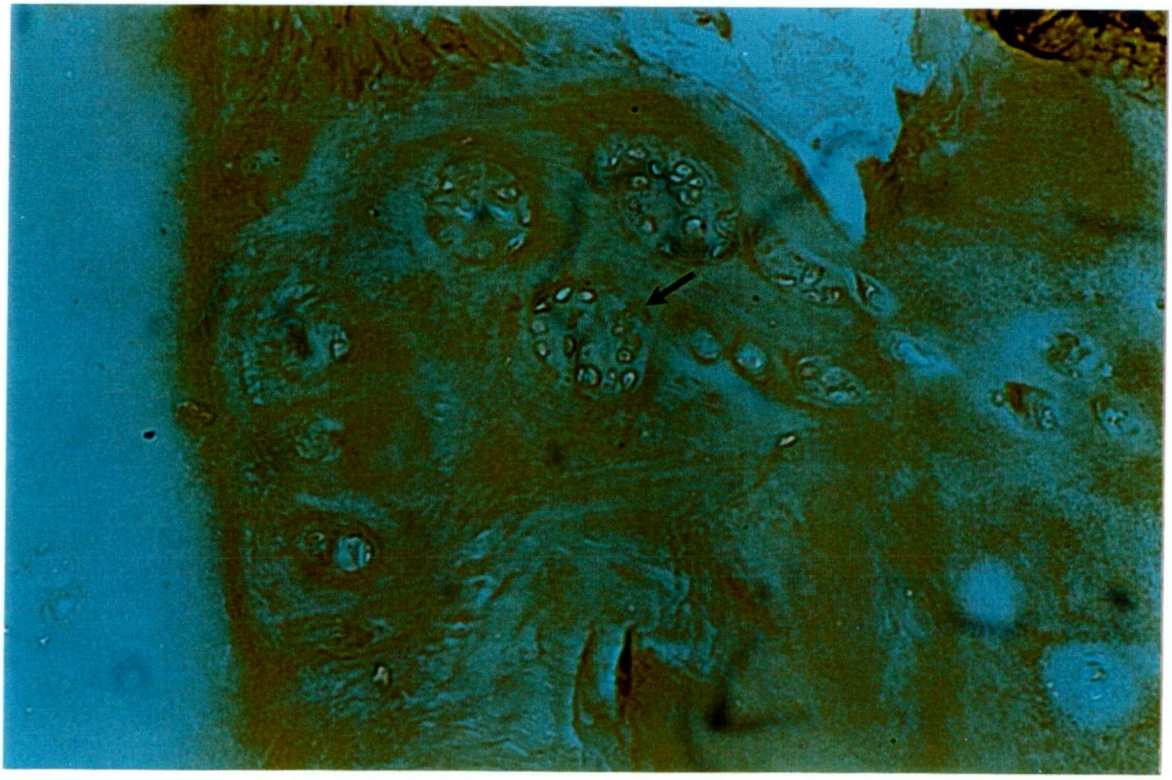


Figure 4-63 PSA Lectin Staining of Clones in Severe OA

Mild staining all parameters in clones. Arrow points to a clone.

4.7.2.3 EPHA

This lectin stains non-bisected and small saccharide sequences. Staining in all five groups was similar.

SURFACE: where present there was moderate to strong staining of the surface.

CHONDROCYTES: there was moderate to strong staining of the cytoplasm and membranes of the cells and clones. All chondrocytes stained in zone I-III whereas in zone IV and the pegs 40-60% of the cells stained.

MATRIX: there was mild to moderate staining of all regions in zones I and II. In zones III and IV pericellular staining was moderate and decreased across the territorial and interterritorial regions (this differentiation was more pronounced in zone IV). In the ageing and OA groups there was virtual loss of staining in zone IV, the changes most pronounced in the territorial and interterritorial matrix. Zone V matrix showed mild staining, pegs did not stain. The periclonal and interclonal areas exhibited mild to moderate staining.

CHONDRO-OSSEOUS JUNCTION: the tidemark did not stain. There was strong staining of vessels, vessel matrix, splits at the tidemark.

SUBCHONDRAL BONE: there was strong staining of both osteocytes and matrix.

NEW CARTILAGE: where present the chondrocyte membrane and matrix showed moderate staining in some of the chondrocytes and there was weak matrix staining.

A summary of the key findings is provided in Table 4-9.

An example of the pattern of staining is seen in Figure 4-64.

Table 4-9 Summary of EPHA lectin histochemistry – Group 2: Complex N-Linked

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CYCM	PC/TM	IM	TD	MA	OS
0 NORM	3	3	3	2	3/3	2/2	2	3/3	2/1	1	3/3	2/1	1	0	3	3
1 EARLY	3	3	3	2	3/3	2/2	2	3/3	2/1	1	3/3	2/1	1	0	3	3
2 AGED	3	3	3	3	3/3	2/2	2	3/3	2/1	1	3/3	1/0	0	0	3	3
3 MOD	3	3	3	2	3/3	2/2	2	3/3	2/1	1	3/3	1/0	0	0	3	3
4 SEVER	3	3	3	2	3/3	2/2	2	3/3	2/1	1	3/3	1/0	0	0	3	3

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was strong CY and CM in all cells.
Matrix: in zones I and II there was moderate staining and zones III and IV, this decreased PC>TM>IM with virtual absence in zone IV in the OA groups.
Tidemark: no staining, osteocytes and bone matrix: strong staining.

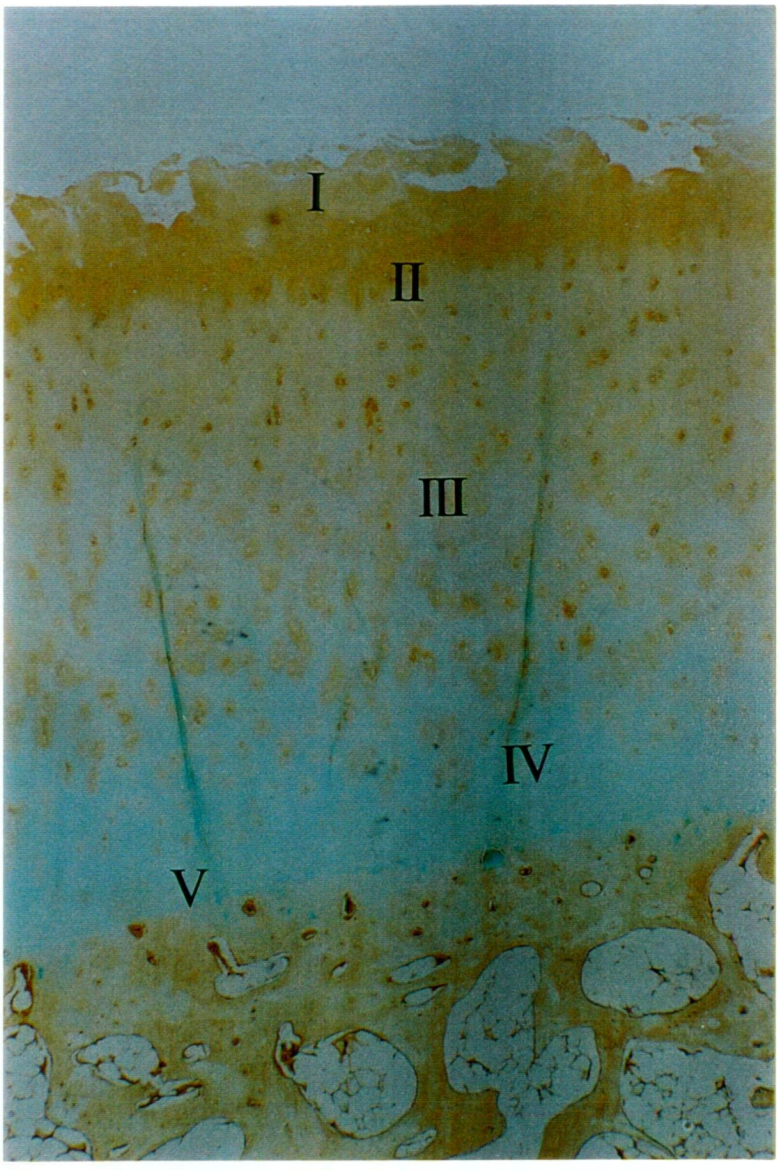


Figure 4-64 EPHA Lectin Staining in Ageing Cartilage

The staining pattern is: chondrocyte mild, matrix in zones I and II moderate and matrix in zones III and IV PC>TM>IM.

4.7.2.4 LPHA

Stains non-bisected, small saccharide, tetra-antennary and sialyl sequences.

SURFACE: mild in normals with a decrease in the other groups.

CHONDROCYTES: in the normal, ageing and early OA groups the cytoplasm and membrane staining patterns were similar: in zones I and II there was mild to moderate staining in up to 40% of chondrocytes, In zones III and IV cytoplasm staining was seen in a decreased percentage of cells and in membrane was absent. In the moderate and severe OA groups, the pattern in zone I and II were similar, in zones III and IV there was a mild increase in the intensity and percentage of cells staining. Peg chondrocytes did not stain. In the clones there was cytoplasm staining in the majority of cells and in membrane in a small percentage of cells.

MATRIX: matrix staining was mild to moderate in all groups in zones I and II with a decrease PC>TM>IM. In these groups in zones III and IV there was a similar graduation in the regions. In the MOA and SOA groups there was absent staining in zones III and IV. Pegs and zone IV matrix did not stain. Periclonal and intraclonal staining was moderate.

CHONDRO-OSSEOUS JUNCTION: no staining of the tidemark. Vessel and vessel matrix staining was mild to moderate in the moderate and severe OA groups and absent in the other groups. Splits stained only in the two more severe groups.

SUBCHONDRAL BONE: osteocyte staining was absent in the normal, ageing, early and mild OA groups and present in a small percentage of the severe OA group. Mild to moderate bone matrical staining was seen throughout.

NEW CARTILAGE: where present there was moderate staining of matrix and cytoplasm (few cases) and absence in cell membranes.

A summary of the key findings is provided in Table 4-10.

Examples of the staining pattern are seen in Figures 4-65 and 4-66.

Table 4-10 Summary of LPHA lectin histochemistry – Group 2: Complex N-Linked

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	3	2	2	2	2/2	2/1	1	2/0	2/1	1	2/0	2/1	1	0	1	0
1 EARLY	2	2	2	2	2/2	2/1	1	2/0	2/1	1	2/0	2/1	1	0	1	0
2 AGED	2	2	2	2	2/2	2/1	1	2/0	2/1	1	2/0	2/1	0	0	1	0
3 MOD	2	2	2	2	2/2	2/1	1	3/0	0/0	0	3/0	0/0	0	0	1	0
4 SEVER	2	2	2	2	2/2	2/1	1	3/0	0/0	0	3/0	0/0	0	0	1	1

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: CY and CM staining was mild in zones I and II in all groups. In zones III and IV CM staining was absent and CY strong in the MOA and SOA groups.

Matrix: moderate all groups I, decreasing PC>TM>IM zone II, III and IV and much more pronounced in severe groups.

Tidemark: absent. Osteocytes: SOA group only. Bone matrix: mild all groups.

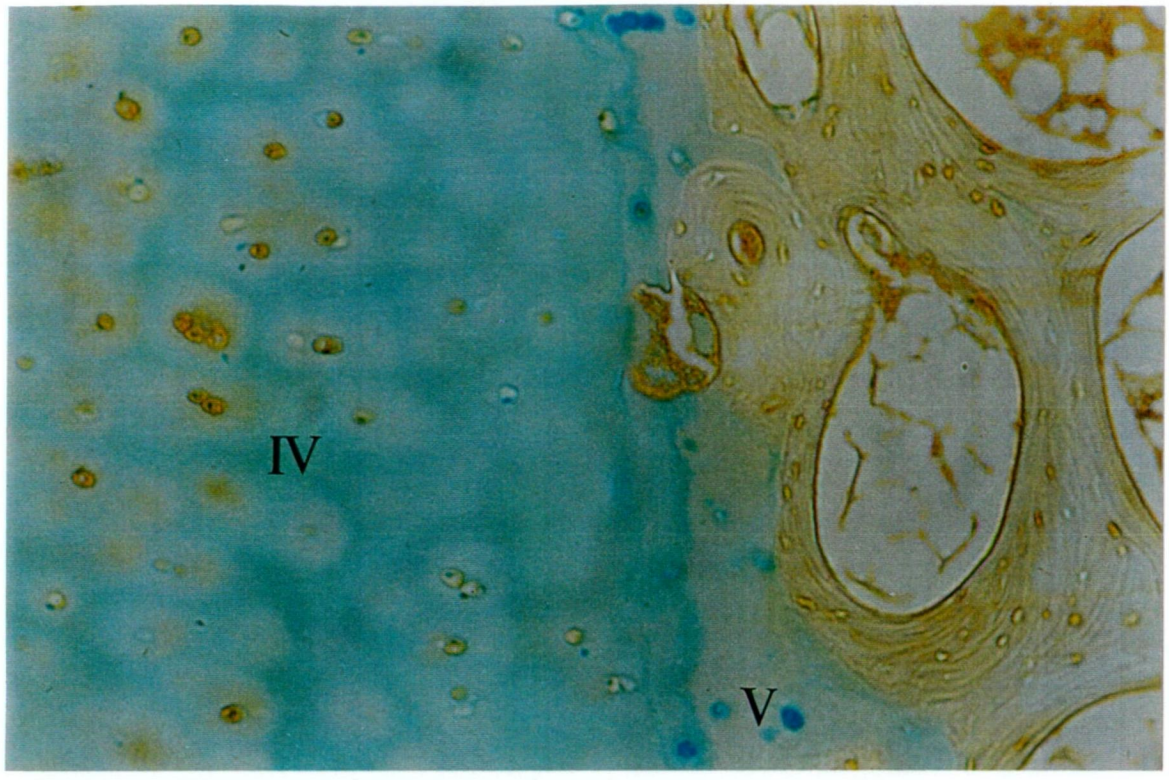


Figure 4-65 LPHA Lectin Staining in Deeper Zones of Normal Cartilage

The staining pattern is: some chondrocytes in zone IV, osteocytes and bone matrix and vessels and vessel matrix.

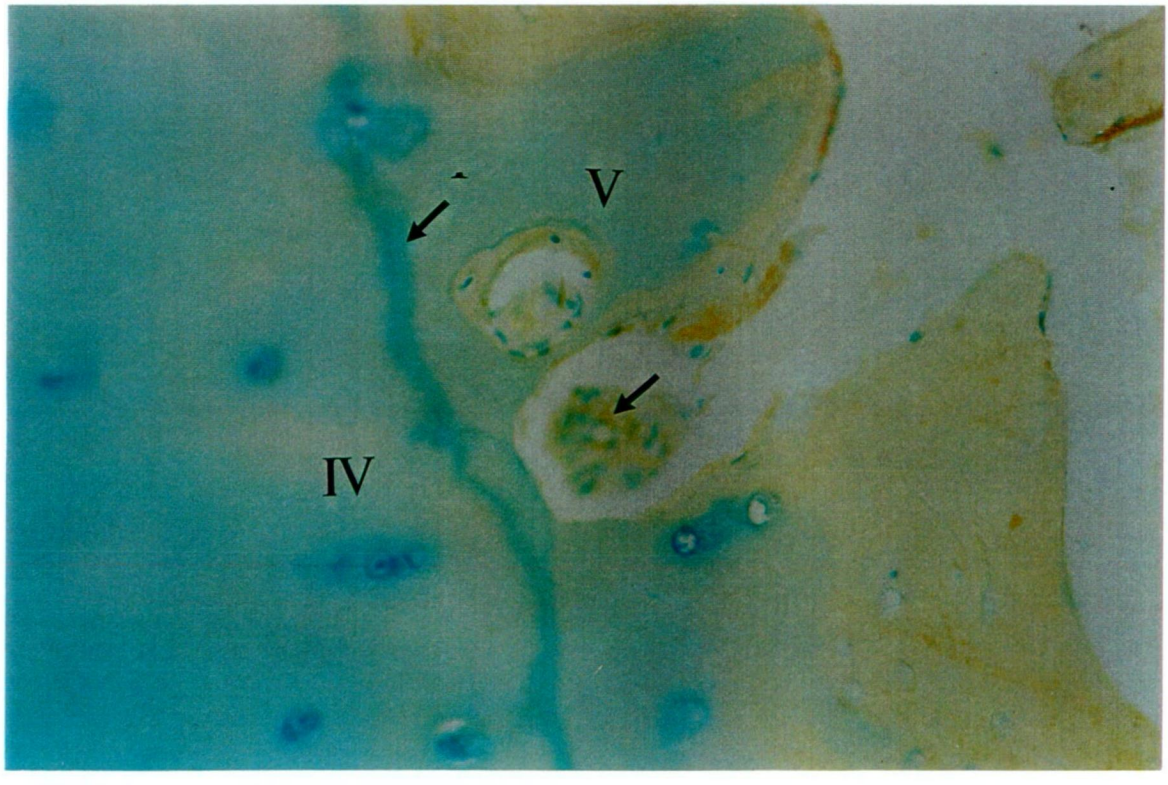


Figure 4-66 LPHA Lectin Stain of Vessels at Tidemark in Moderate OA

There is moderate staining of vessels extending through the calcified cartilage to the tidemark. The upper arrow points to the tidemark and the lower arrow to vessels.

4.7.3 GROUP 3 – N-acetyl lactosamine, di-N-acetyl chitobiose

The lectins in the groups are STA, DSA and WGA. DSA has some staining affinity for group 4 lectins which stain β galactose and WGA with Group 7 lectins as it stains sialyl residues.

4.7.3.1 DSA

SURFACE: moderate to strong surface staining in all groups.

CHONDROCYTES: in all groups, there was moderate membrane and cytoplasm staining in all zones and regions. In the normal, ageing and severe OA groups the majority of cells in zones I-III stain whereas only 40-60% do in zone IV. In the early and mild OA groups there was a slightly greater percentage of zone IV cells staining at between 60-80%. Only 20-40% of peg chondrocytes were staining whereas the majority of clones stained.

MATRIX: in the first few groups there was mild to moderate staining of all regions in zones I-III with zone IV showing PC<TM+IM. In the severe OA group there was a marked decrease in staining in zone III and a complete absence in zone IV. In zone V staining was mild in the normal and ageing groups, moderate in the early OA group and absent in the last two groups. The pegs did not stain in any groups. The intra and periclonal regions showed mild to moderate staining.

CHONDRO-OSSEOUS JUNCTION: tidemark staining was mild in the normals, just present in the ageing group, mild to moderate in the early OA group and absent in the last two groups. Staining of vessels, vascular matrix and splits was moderate.

SUBCHONDRAL BONE: strong osteocyte and matrix staining was seen.

NEW CARTILAGE: where present there was moderate cytoplasm and membrane staining in the majority of cells with patchy matrix staining.

A summary of the key findings is provided in Table 4-11.

Examples of the staining patterns are seen in Figures 4-67 to 4-69.

Table 4-11 Summary of DSA lectin histochemistry – Group 3: N-Acetyl Lactosamine, Group 4: β - Galactose

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	3	2	2	2	2/2	2/1	1	2/2	2/1	1	2/1	2/1	1	1	3	3
1 EARLY	3	2	2	2	2/2	2/1	1	2/2	2/1	1	2/2	2/1	1	1	3	3
2 AGED	3	2	2	2	2/2	2/1	1	2/2	2/1	1	2/2	2/1	1	2	3	3
3 MOD	3	2	2	2	2/2	2/1	1	2/2	2/1	0	2/2	2/1	0	2	3	3
4 SEVER	3	2	2	2	2/2	2/1	1	2/2	2/1	0	2/2	0/0	0	2	3	3

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: moderate CM and CY present in all zones in all groups.
Matrix: moderate in zone I decreasing across regions in zone I-IV PC>TM>IM and in severe groups in III and IV there is no staining.
Tidemark: staining intensity increases across groups. Osteocytes and bone matrix: strong staining.

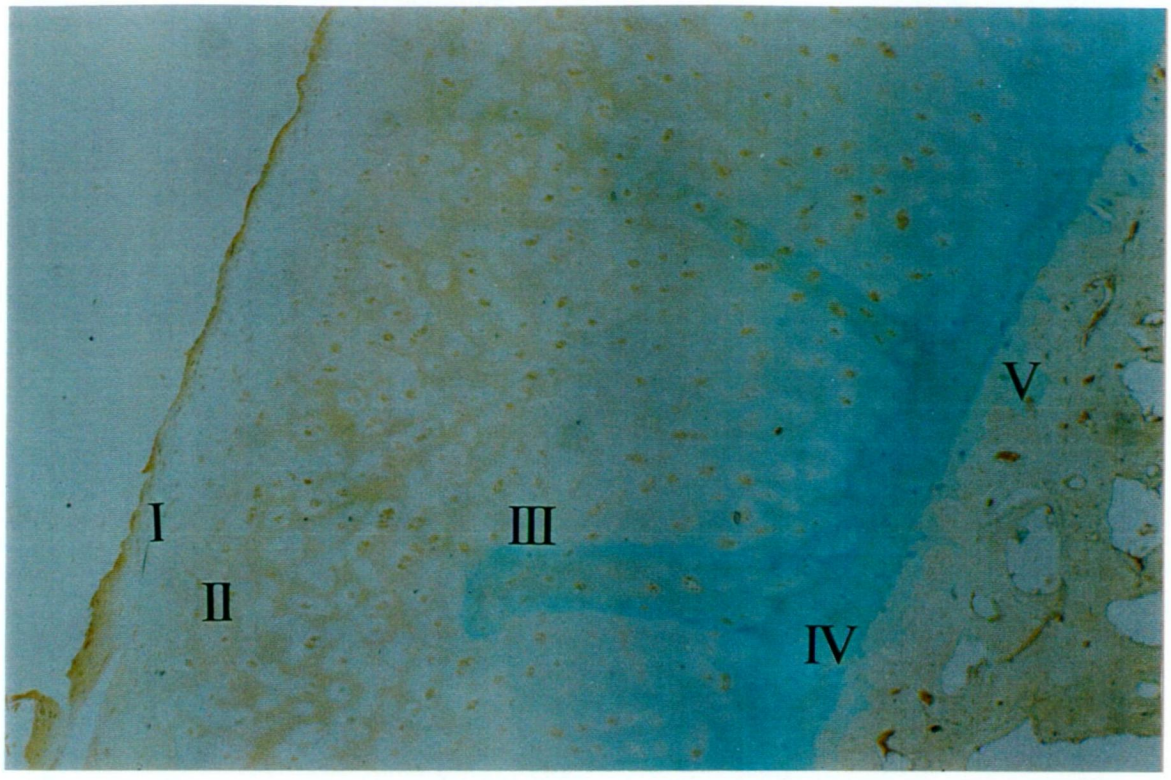


Figure 4-67 DSA Lectin Staining in Normals

Staining pattern: moderate surface, chondrocytes all zones and matrical staining TM and IM > PC.

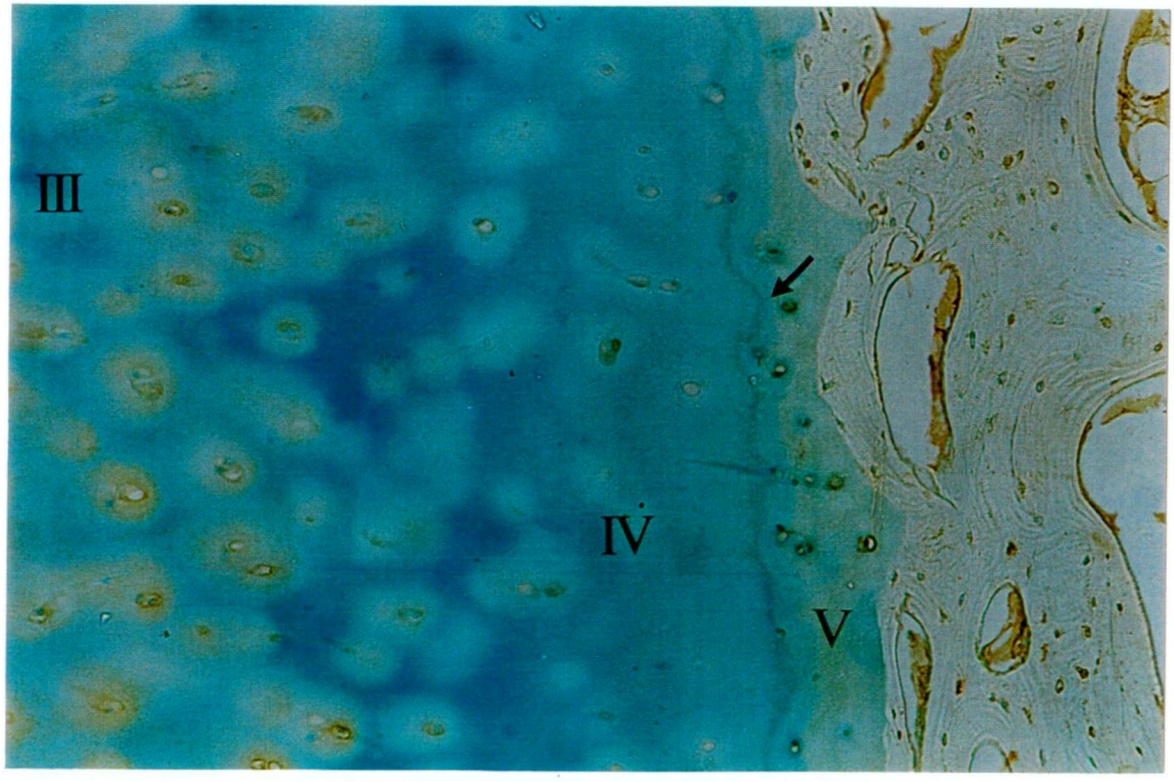


Figure 4-68 DSA Lectin Staining of Tidemark in Early OA Group

Main feature is mild staining of the tidemark in early OA. Arrow points to the tidemark.

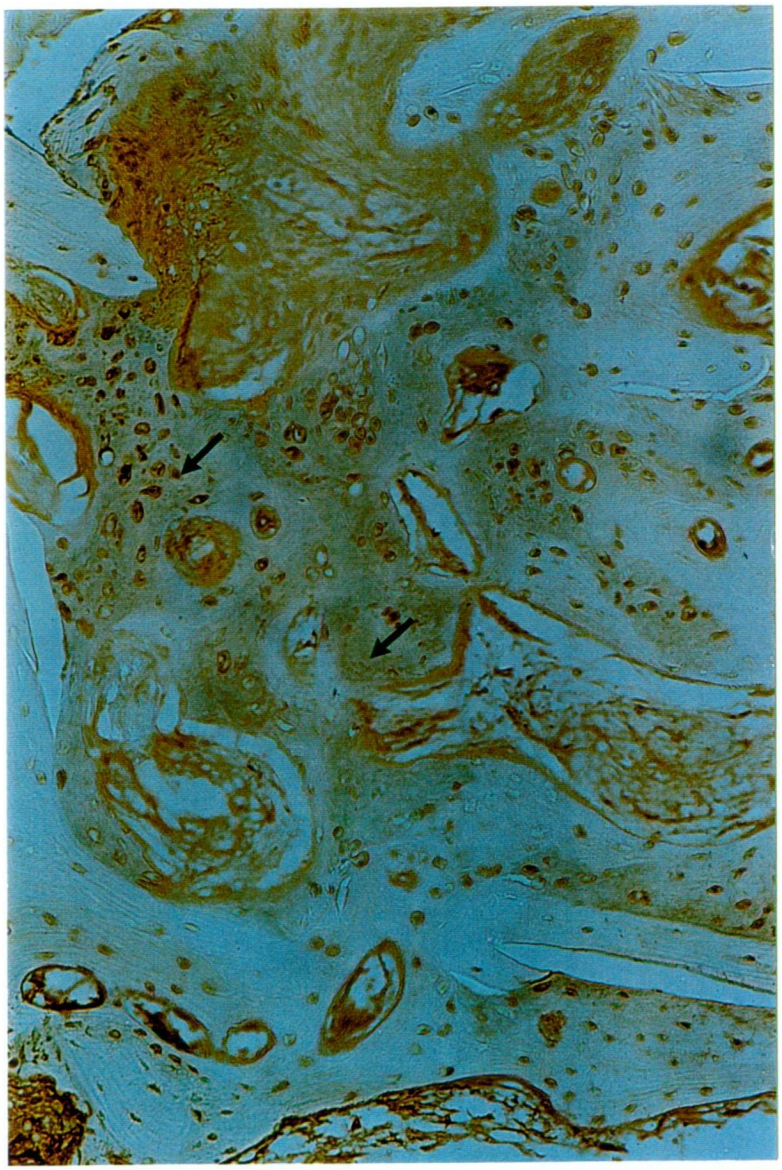


Figure 4-69 DSA Lectin Staining in Severe OA of Subchondral Bone

Subchondral bone shows prominent osteocyte and matrix staining. Upper arrow points to osteocytes and the lower arrow to bone matrix

4.7.3.2 STA

Results of staining in the five groups were:

SURFACE: mild to moderate staining of the surface was seen in the normal, ageing and early OA groups with some loss of staining in the mild OA groups.

CHONDROCYTES: in all groups there was mild to moderate staining of the cytoplasm and membrane in all zones and groups. The majority of cells stained in all groups apart from the severe OA group where the percentage dropped from nearly 90-100% to 40-60%. The staining intensity of the cytoplasm and membranes in peg chondrocytes was very strong with between 60% of cells staining. There was moderate cytoplasm and membrane staining of small clones and strong in large clones.

MATRIX: in the normal group there was moderate staining in zones I-III in all regions and in zone IV pericellular staining was moderate, whereas territorial and interterritorial staining was weak. The staining pattern in the ageing and early OA groups was the same apart from being mild in zone IV. In the mild to severe OA groups there was a decrease in staining in all zones and regions with PC>TM>IM. Zone V and pegs did not stain. Periclonal and intraclonal regions showed mild staining.

CHONDRO-OSSEOUS JUNCTION: tidemark staining was absent in the normal, moderate and ageing OA groups, in early OA was moderate in under 20% of cases and in the severe OA group was moderate in up to 50% of cases. Vessels, vessel matrix and tidemark splits showed moderate to strong staining.

SUBCHONDRAL BONE: strong in osteocytes and matrix.

NEW CARTILAGE: where present there was moderate to strong cytoplasm and membrane staining in the majority of cells with mild to moderate matrix staining.

A summary of the key findings is provided in Table 4-12.

An example of the staining pattern is seen in Figure 4-70.

Table 4-12 Summary of STA lectin histochemistry – Group 3: N-Acetyl Lactosamine

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	3	2	2	2	2/2	2/2	2	2/2	2/2	2	2/2	2/1	1	0	3	3
1 EARLY	3	2	2	2	2/2	2/2	2	2/2	2/2	2	2/2	2/1	1	0	3	3
2 AGED	3	2	2	2	2/2	2/2	1	2/2	2/2	1	2/2	2/1	1	2	3	3
3 MOD	2	2	2	2	2/2	2/2	1	2/2	2/1	0	2/2	2/1	0	0	3	3
4 SEVER	3	2	2	2	2/2	2/1	1	2/2	2/1	0	2/2	2/1	0	2	3	3

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: CY and CM showed moderate staining in all zones and groups.
Matrix: was moderate in zone I and decreased PC>TM>IM across regions in zone I –IV the greatest differentiation being in the severe groups.
Tidemark: staining in EOA and MOA groups. Osteocytes and bone matrix stain strongly.

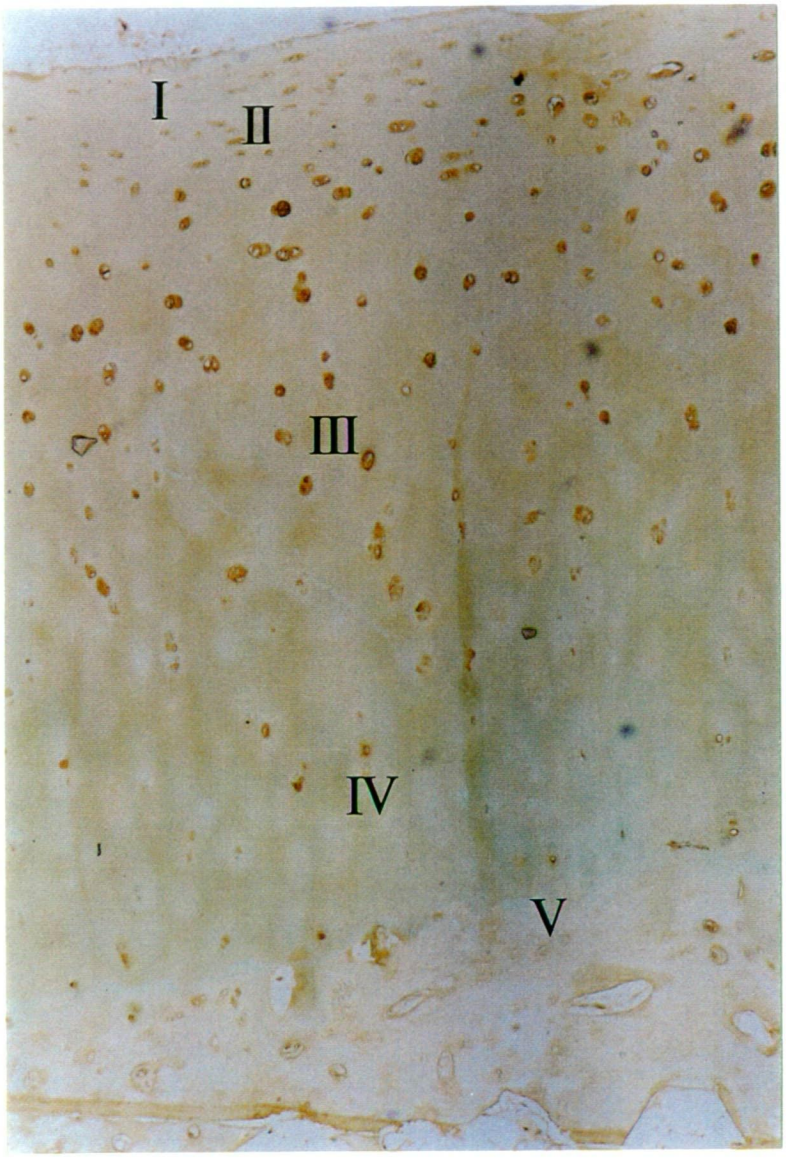


Figure 4-70 STA Lectin Staining in Normal

Prominent chondrocyte and matrical staining.

4.7.3.3 WGA

SURFACE: there was mild to moderate surface staining in all groups.

CHONDROCYTES: in the normals, there was moderate staining in most cells in all zones and in the pegs. In ageing and early OA there was a decrease in staining in zones I/II and in the number of positive cells. In the moderate and severe OA groups staining was absent in zones I/II whereas in zones III , IV and pegs there was moderate staining in less cells. Chondrocyte clones did not stain in ageing and early OA, whereas in moderate and severe OA there was moderate staining of 40% and 80-100% of cells respectively.

MATRIX: there was moderate staining of all regions in the first three groups in zones I/II whereas it was only mild in zones III/IV (PC>TM>IM). With increasingly severe OA there was a decrease in staining in zones I/II and an absence in III and IV, most pronounced in the moderate group. Zone V staining was absent in the normals and mild in the other groups. Intra- and periclonal staining was absent in the ageing and early OA groups and moderate in the other groups.

CHONDRO-OSSEOUS JUNCTION: tidemark was negative. Vessel matrix and splits were negative in the normals and stained increasingly in the other groups.

SUBCHONDRAL BONE: moderate osteocyte staining in all groups. Matrix was negative in the normals and ageing groups and present with increasing intensity across the OA groups.

NEW CARTILAGE: Moderate cytoplasm, membrane and matrix staining.

ENZYMATIC DEGRADATION: ***Neuraminidase*** – in all groups there was a uniform increase in matrix and chondrocyte staining in all zones and regions.

Aryl sulfatase – in all groups there was a very marked increase in membrane staining and to a lesser extent cytoplasm. These changes were most pronounced in the normal, ageing and early OA groups. There was an increase in matrix staining in all groups.

A summary of the key features is provided in Table 4-13.

Examples of the staining patterns are seen in Figures 4-71 to 4-76.

Table 4-13 Summary of WGA Lectin Histochemistry – Group 3: N-Acetyl Lactosamine, Group 7: Sialic acid

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	2	2	2	2/2	2/2	2	2/2	2/1	1	2/2	2/1	1	0	0	2
1 EARLY	2	1	1	2	1/1	2/2	2	1/1	2/1	1	1/1	2/1	1	0	0	2
2 AGED	2	1	1	2	1/1	2/2	2	1/1	2/1	1	1/1	2/1	1	0	1	2
3 MOD	2	0	0	1	0/0	1/1	1	0/0	1/0	0	0/0	1/0	0	0	2	2
4 SEVER	2	0	0	1	0/0	1/1	1	0/0	1/0	0	0/0	1/0	0	0	2	2

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: cytoplasm and cell membrane staining was strongest in the normals and decreased across the groups.
Matrix: staining was strongest in the upper zones, decreased across the groups and across the regions P<TM/IM.
Chondrocyte clones: stained in the severe OA group only.
Tidemark: did not stain; osteocytes stain in OA group; bone matrix stains all groups.
Neuraminidase: chondrocytes, matrix and clones all showed increased staining. Differentiation between regions was difficult.
Aryl sulfatase: increased membrane and to a lesser extent cytoplasm staining most pronounced in the OA groups. The matrix showed a mild overall increase in staining.

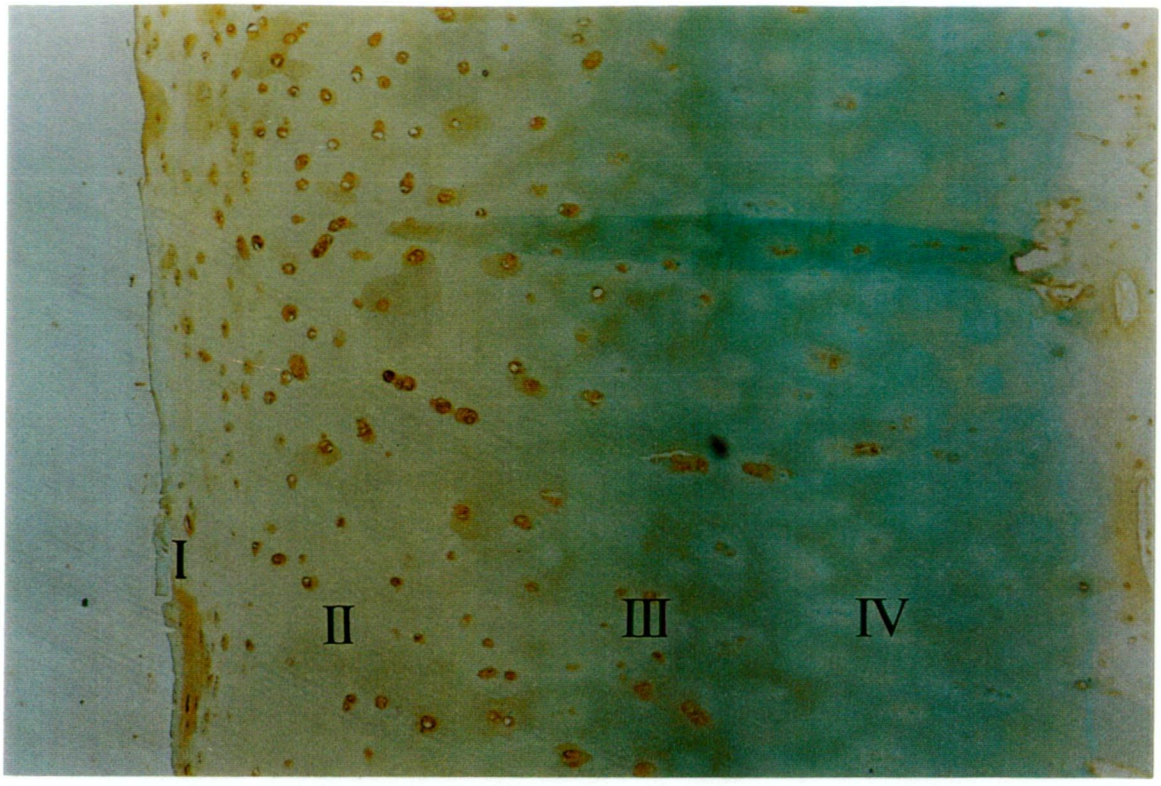


Figure 4-71 WGA Lectin Staining in Normals

Staining pattern: chondrocytes, osteocytes and matrix with PC>TM>IM especially zones III and IV.

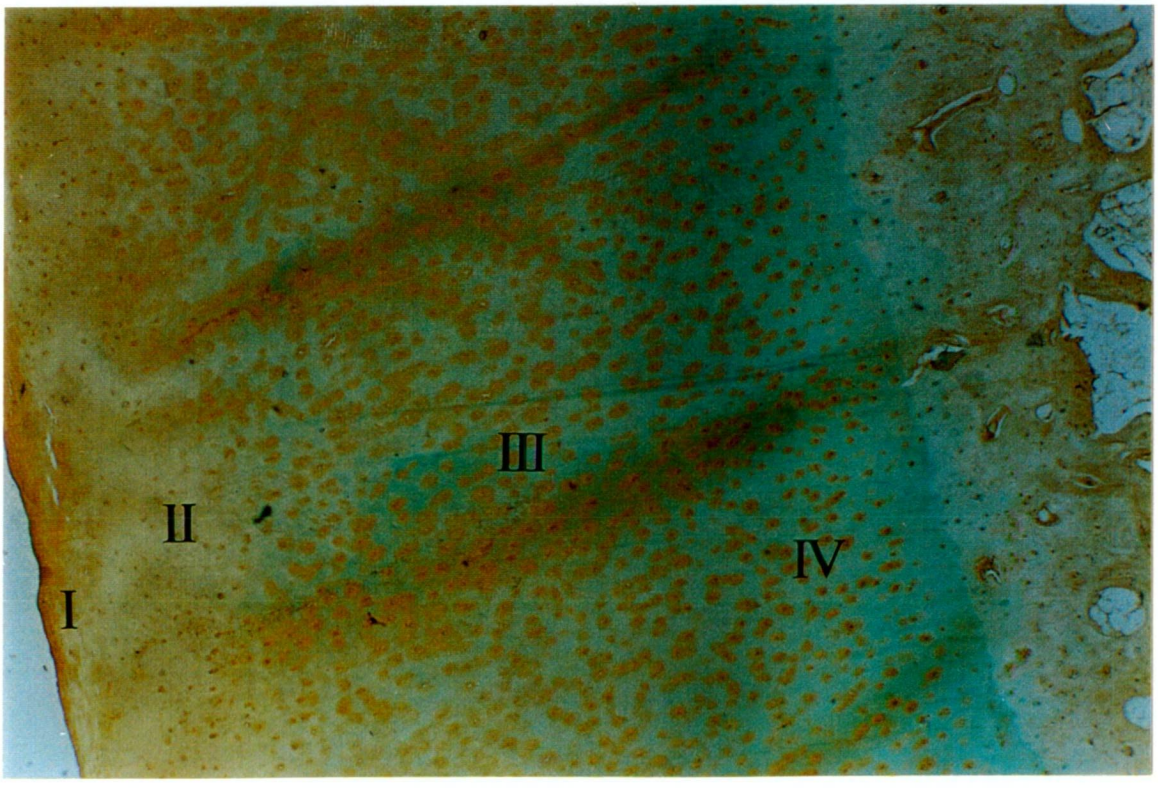


Figure 4-72 WGA Lectin Staining in Normals Following Neuraminidase

Same pattern as normals but overall increase in staining intensity with neuraminidase digestion.

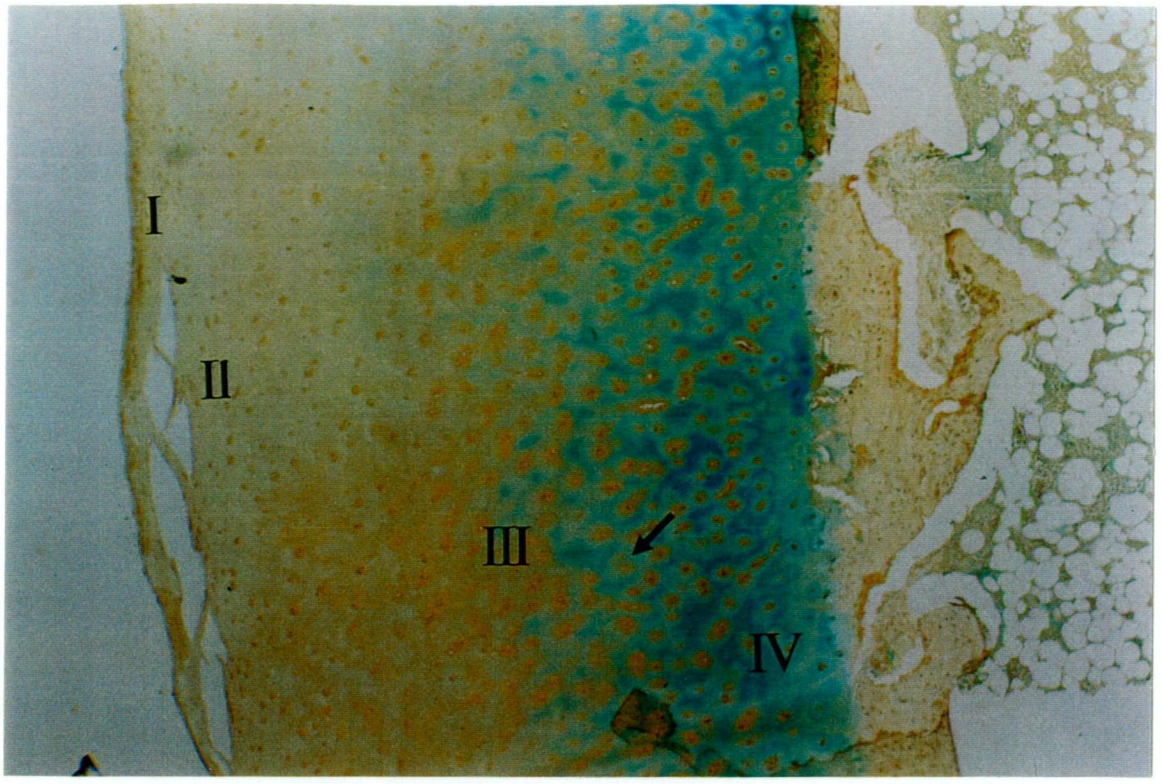


Figure 4-73 WGA Lectin Normals After Aryl Sulfatase
Marked increase in chondrocyte staining especially cell membranes following neuraminidase digestion. Arrow points to chondrocytes in zone IV.

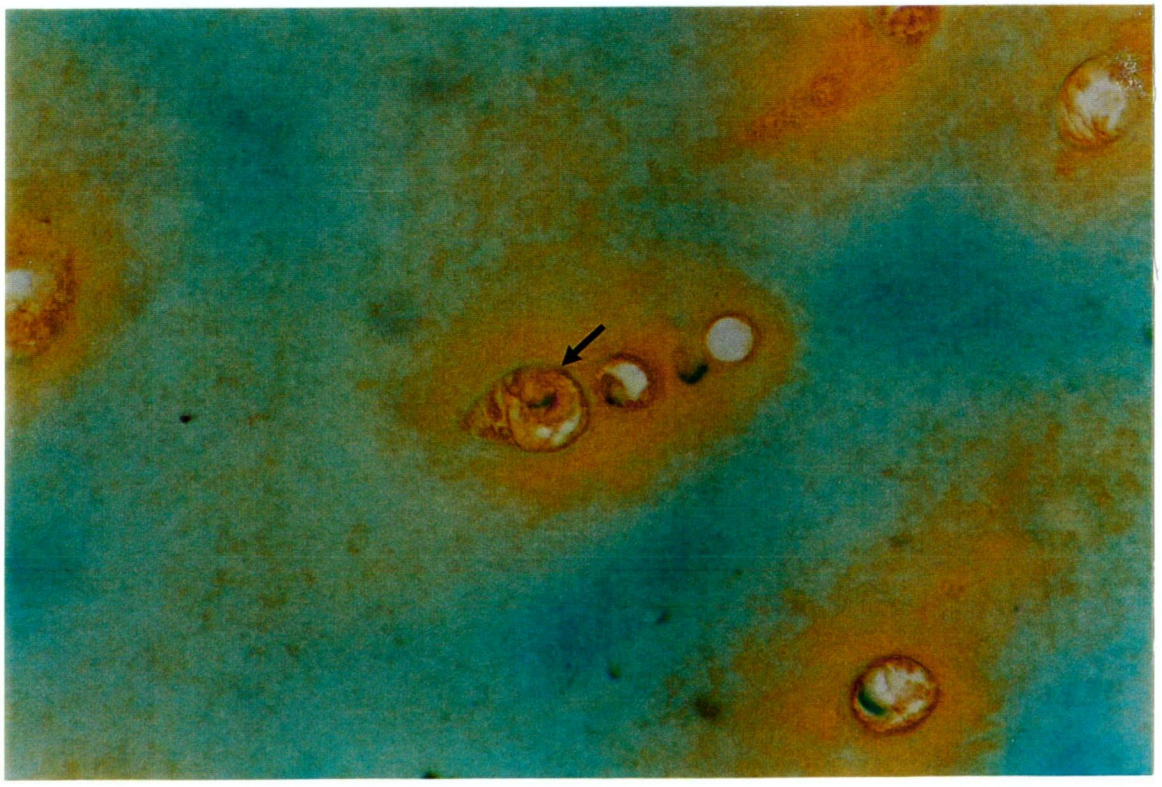


Figure 4-74 WGA Lectin in Normals to show Chondrocytes After Aryl Sulfatase

Very prominent cell membrane staining is seen and cytoplasmic and pericellular staining is increased. Arrow points to this region.

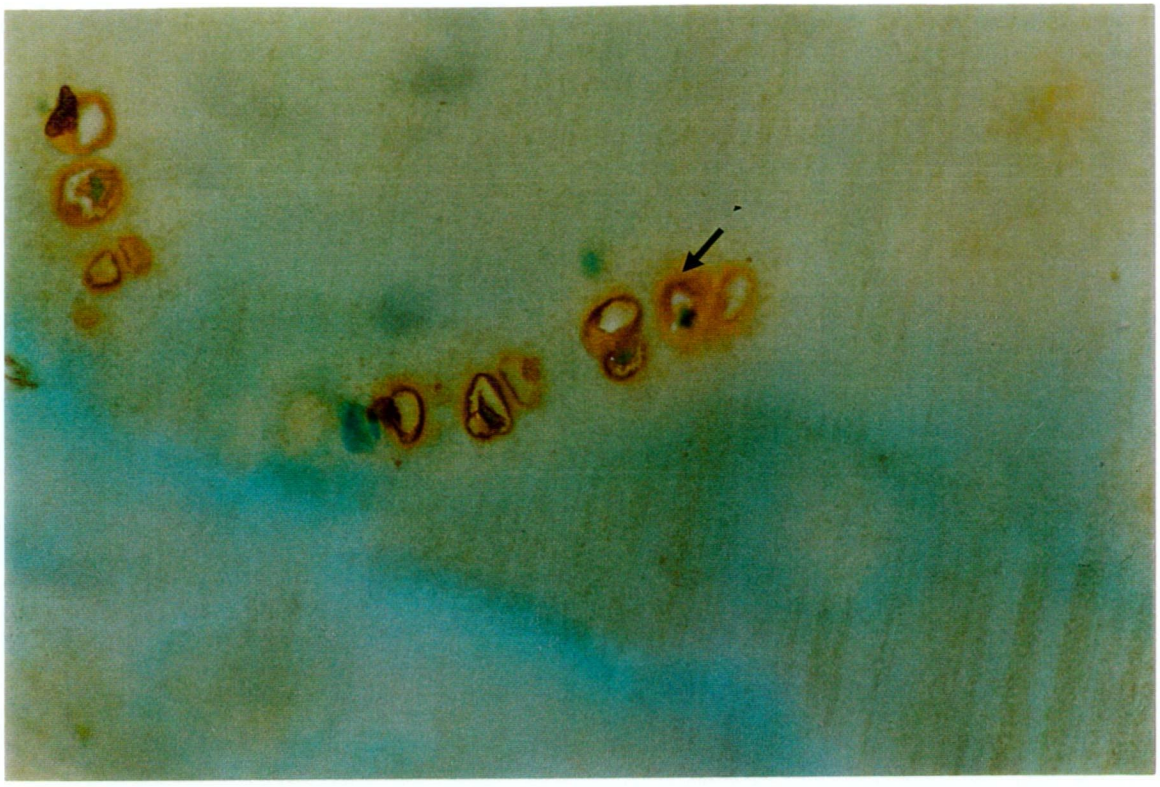


Figure 4-75 WGA Staining of Pegs in Normals with Neuraminidase

Very prominent staining of chondrocytes in pegs (arrow) with neuraminidase digestion.

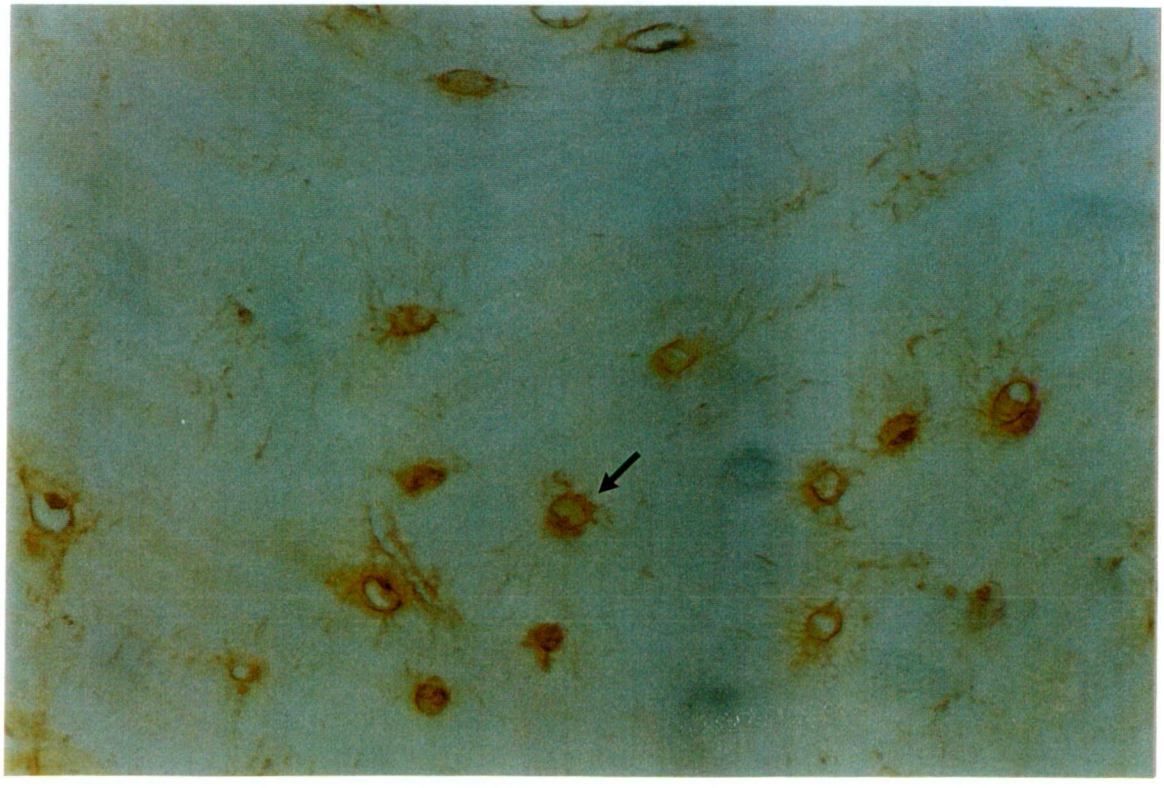


Figure 4-76 WGA Staining of Osteocytes in MOA

Osteocytes and their dendritic processes are clearly defined. Arrow points to an osteocyte.

4.7.4 GROUP 4 - β galactose

This group of lectins is represented by MPA, ECA and CTA.

4.7.4.1 MPA

MPA has a preferred specificity for β-galactose but also has affinity with Group 5.

SURFACE: There was moderate surface staining.

CHONDROCYTES: there was moderate cytoplasm and membrane staining in the normal and ageing groups in all zones, the intensity being less in the deep zones. Mild staining was seen in the OA groups and the intensity was again less in the deep zones. In normals, ageing and early OA groups in zones I/II, 80-100% of cells stain and in zones III/IV 60-80%. In the severe groups only a few percent stain in zones I-IV.
Moderate peg chondrocyte staining was present in a few cases in the normals in 20-40% of cells.
There was mild staining of cytoplasm and membrane of clones in a few cases in the ageing group and none in the early OA group. In the moderate OA group there was moderate membrane staining in 60-80% of cells and this was absent in the cytoplasm. In the severe group there was staining of cytoplasm and membrane in 60-80% of cells in about half the cases.

MATRIX: in the normal group there was moderate staining in zone I. In zones II/III PC>TM>IM and in zone IV there was PC only. A similar pattern was seen in the ageing and early OA groups with stronger staining in the territorial and interterritorial regions. In the severe OA groups staining was moderate in zone I, mild in zone II, pericellular only in zone III and absent in zone IV. Zone V matrix staining was strong and intra- and periclonal moderate.

CHONDRO-OSSEOUS JUNCTION: The tidemark stained, vessels, vessel matrix and splits were negative in the normals, mild in a few cases only in the ageing group and moderate in the OA groups.

SUBCHONDRAL BONE: strong staining of osteocytes and bone matrix.

NEW CARTILAGE: cytoplasm and membranes moderate, matrix absent.

ENZYMATIC DEGRADATION:

Neuraminidase – in all zones in all cases there was an increase in cytoplasm, membrane and matrix staining (TM + IM now staining). Osteocyte and tidemark staining was stronger.

β Galactosidase – normal and ageing groups unaltered. In the OA groups there was less staining of chondrocytes and matrix whereas that of the tidemark was more.

β Elimination – in all groups there was overall decreased chondrocyte staining. Matrix and tidemark staining was increased. Osteocytes were unaltered.

A summary of the key features is provided in Table 4-14.
Examples of the staining patterns are seen in Figs 4-77 to 4-84.

Table 4-14 Summary of MPA lectin histochemistry - Group 4: β - Galactose, Group 5: N-Acetyl Galactosamine

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	2	2	2	2/2	2/1	1	2/2	2/0	0	1/1	2/0	0	2	4	4
1 EARLY	2	2	2	2	2/2	2/1	1	2/2	2/0	0	1/1	2/0	0	2	4	4
2 AGED	2	2	2	2	2/2	2/1	1	2/2	2/0	0	1/1	2/0	0	2	4	4
3 MOD	2	2	2	2	2/2	2/2	2	2/2	2/0	0	1/1	0/0	0	2	4	4
4 SEVER	2	2	2	2	2/2	2	2/2	2/2	2/0	0	1/1	0/0	0	2	4	4

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was moderate cytoplasm and membrane stains in all groups in zones I and II with only mild staining in zone IV.
Matrix: there was moderate or mild staining in all groups in all regions in zones I and II, pericellular staining remains in zone III and is absent in OA groups in zone IV. Territorial and interterritorial staining was absent in zones III and IV.
Tidemark, osteocytes and bone matrix all show strong staining.
Neuraminidase: cytoplasm, membranes, matrix (all regions) and osteocytes showed increased staining.
β galactosidase: normals and ageing unaltered. OA groups showed decreased staining.
β elimination: chondrocytes staining was increased. Matrix and tidemark staining was increased.

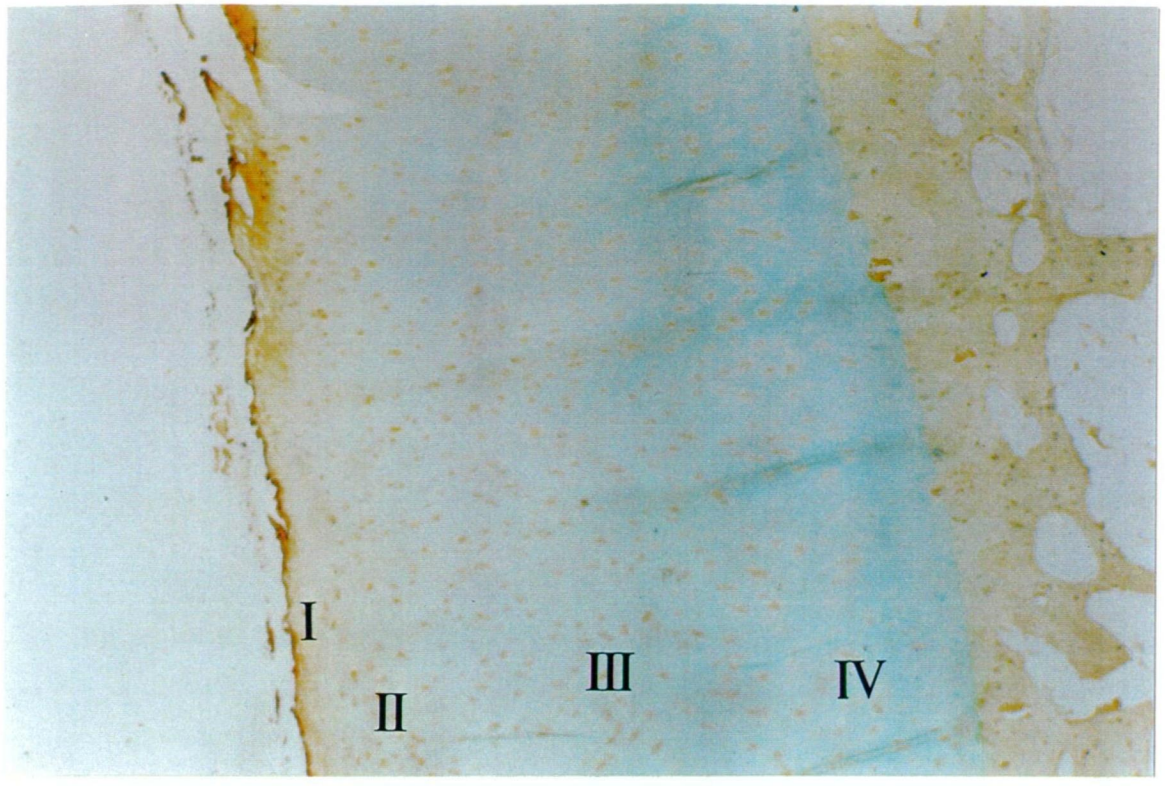


Figure 4-77 MPA Lectin Staining in Normal Cartilage

The staining pattern was moderate on the surface, in chondrocytes and in the matrix moderate at the surface and decreased into the deeper zones.

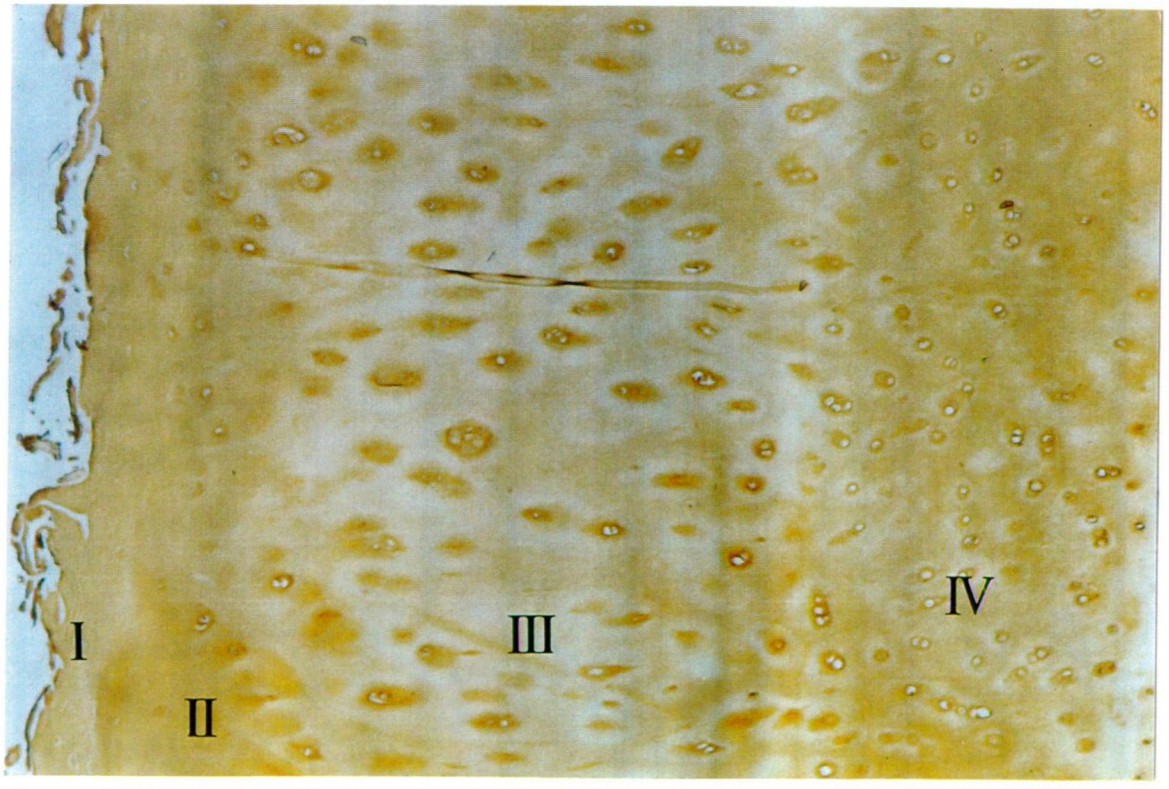


Figure 4-78 MPA Lectin Staining in Normal Cartilage with Neuraminidase

The staining pattern was increased across all parameters.

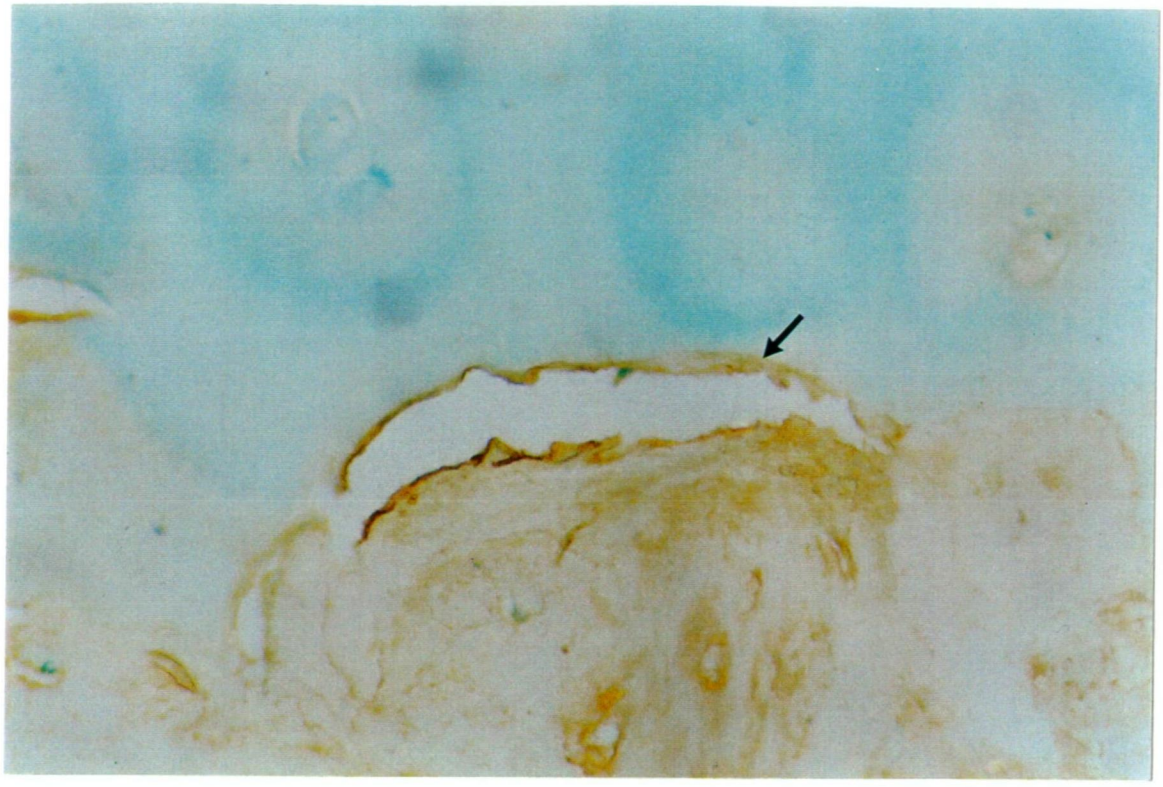


Figure 4-79 MPA Lectin in Moderate OA at the Tidemark

Horizontal split at tidemark with strong edge staining. Arrow points to a split.

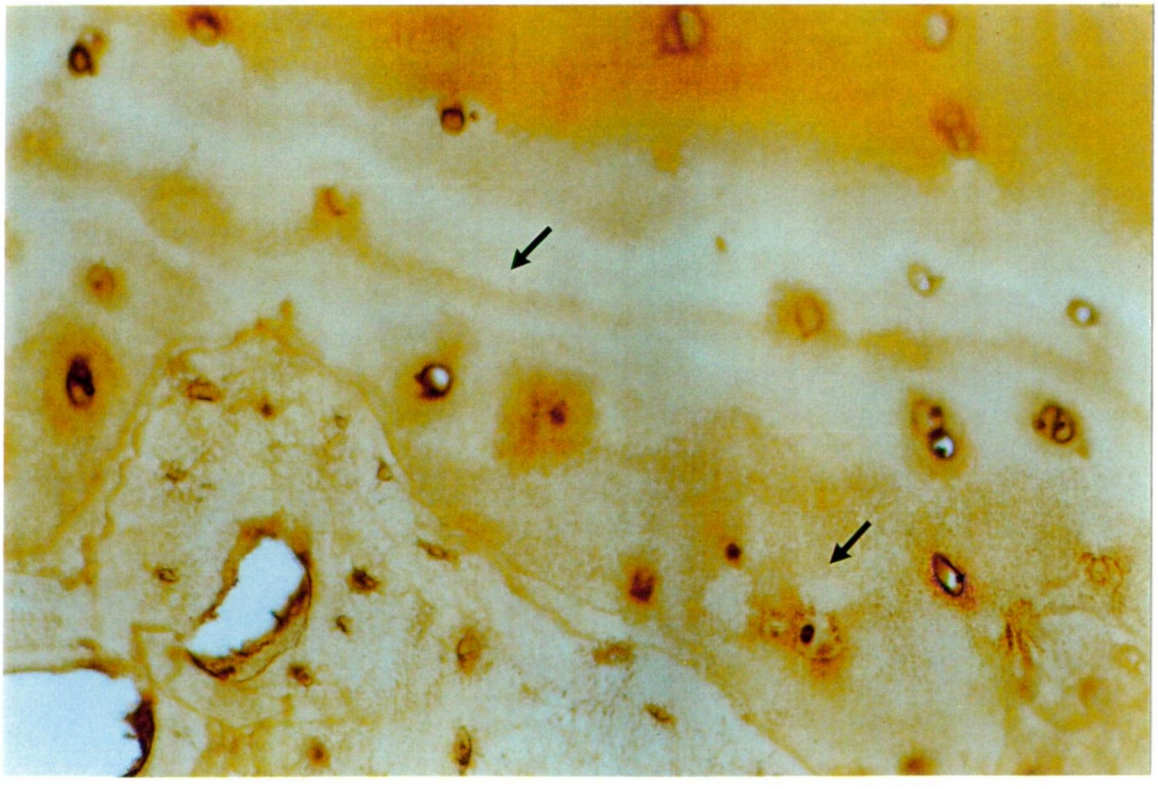


Figure 4-80 MPA Lectin in Moderate OA with Neuraminidase

Prominent tidemark staining (upper arrow) with granular staining of the underlying calcified cartilage (lower arrow).

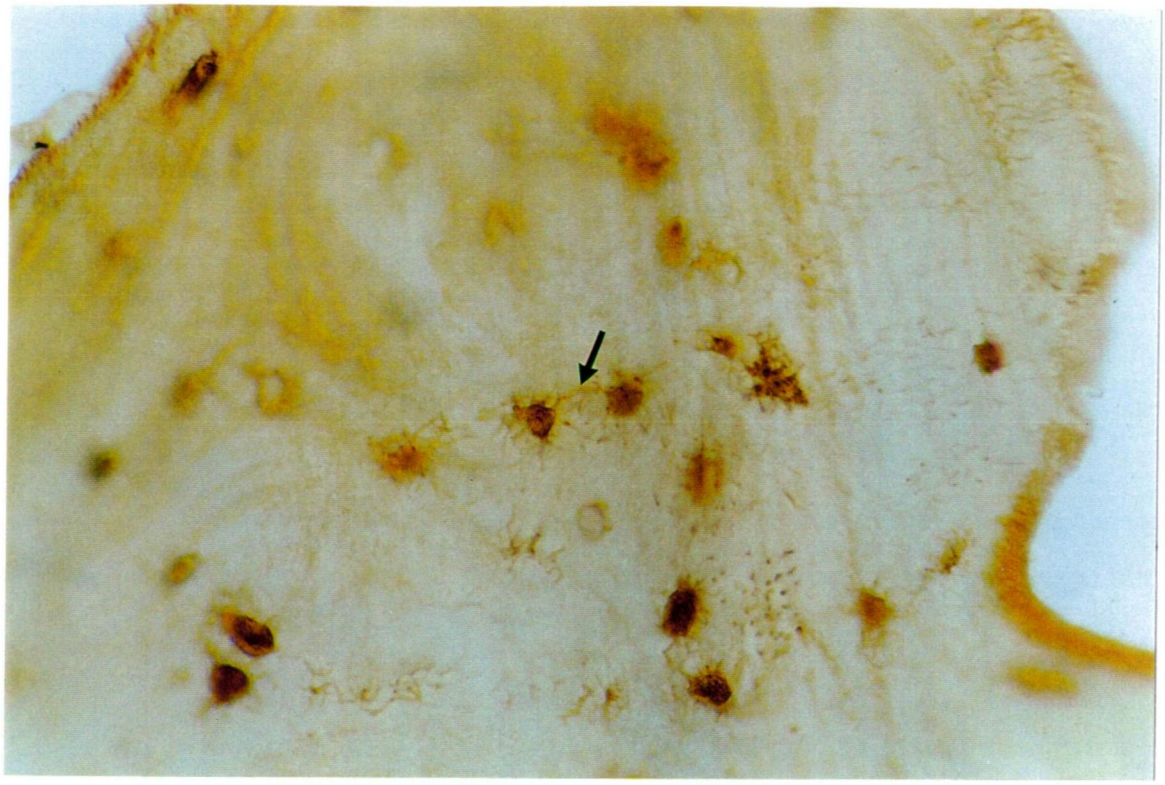


Figure 4-81 MPA Lectin Staining in Bone – Severe OA with Neuraminidase

Very prominent osteocytes and dendritic processes. Arrow points to osteocytes.

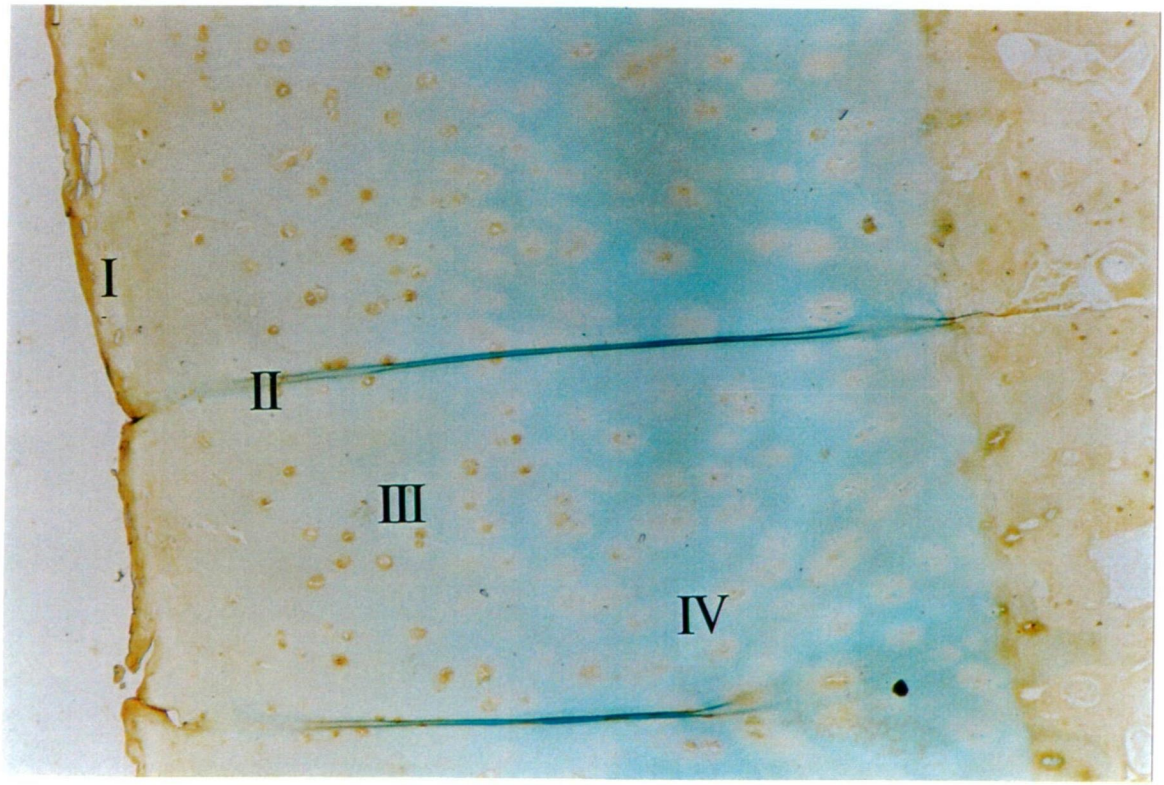


Figure 4-82 MPA Lectin Stain in Normals with β Galactose

Same pattern of staining as for MPA lectin alone.

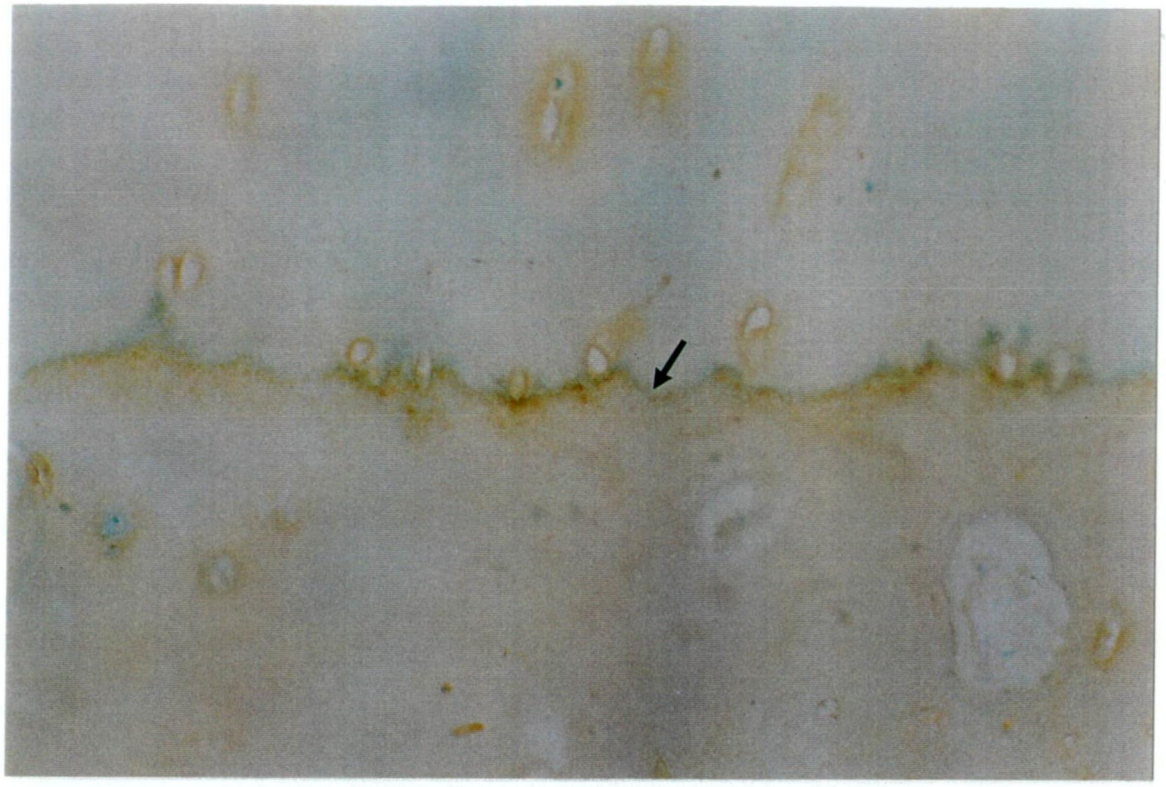


Figure 4-83 MPA Lectin in Normals at the Tidemark with β elimination

A very prominent granular tidemarks seen. Arrow points to the tidemark.

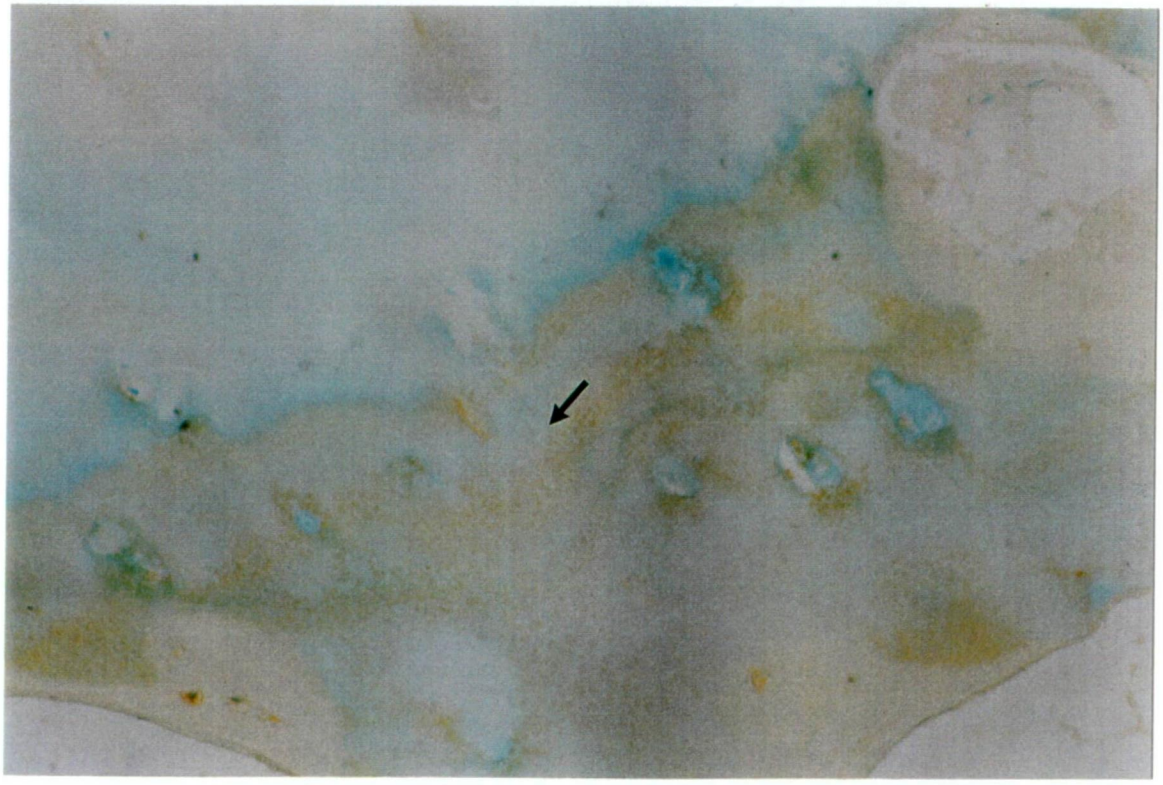


Figure 4-84 MPA Lectin in Moderate OA at the Tidemark with β elimination

Marked granular staining of triplicated tidemark. Arrow points to this region.

4.7.4.2 ECA

This lectin is specific for β-galactose. The results of staining in the five groups were:

SURFACE: weak surface in the normal group only.

CHONDROCYTES: in both normal and ageing groups there was mild cytoplasm and membrane staining in up to 40% of cells in zones I and II, this was absent in the OA groups. In the normal, ageing and early OA groups there was moderate cytoplasm and membrane staining in zones III and IV in up to 60% of cells. In the moderate OA group the pattern was the same in an increased number of cells in zones III and IV. In the severe OA group staining was mild in less than 20% of cells in zone III and absent in zone IV. Peg chondrocyte cytoplasm and membrane show moderate staining in a small percentage of cells in all groups. Clones where present showed moderate staining in a small percentage of cells.

MATRIX: mild to moderate zone I and II staining slightly stronger in the ageing and early OA groups. Mild zone III staining in all regions. Zone IV and V staining was absent. Pericl̨onal and intraclonal matrix stained in some cells.

CHONDRO-OSSEOUS JUNCTION: no staining of parameters.

SUBCHONDRAL BONE: mild osteocyte and bone matrix staining.

NEW CARTILAGE: mild to moderate cytoplasm and membrane staining in 20-40% of cells with no matrix staining.

ENZYMATIC DEGRADATION:

Neuraminidase: staining pattern increased in chondrocytes, clones and matrix in all groups. In the OA groups vessels, matrix and tidemark splits stain.

Fucosidase: there was no change in the normals and ageing group. In the OA group there was increased cell membrane staining particularly in zones III and IV.

Fucosidase and neuraminidase: show the combined effects of two enzymes.

β elimination: all groups in all zones showed increased matrix staining PC>TM>IM. Chondrocytes showed diminished staining.

A summary of the key features is provided in Table 4-15.

Examples of the staining patterns are seen in Figs 4-85 to 4-90.

Table 4-15 Summary of ECA lectin histochemistry – Group 4: β - Galactose

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	1	1	1	2	1/1	2/2	2/2	2/2	1/1	1	2/2	0/0	0	0	1	2
1 EARLY	0	0	0	2	1/1	2/2	2/2	2/2	1/1	1	2/2	0/0	0	0	1	2
2 AGED	0	1	1	2	1/1	2/2	2/2	2/2	1/1	1	2/2	0/0	0	0	1	2
3 MOD	0	0	0	2	2/2	2/2	2/2	2/2	1/1	1	2/2	0/0	0	0	1	2
4 SEVER	0	0	0	2	2/2	2/2	2/2	2/2	1/1	1	0/0	0/0	0	0	1	2

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was mild to moderate chondrocyte CM and CY staining in all groups in all regions.
Matrix: In all groups staining was moderate in zones I and II, mild in zone III and absent in zone IV.
Tidemark does not stain; osteocytes and bone matrix show mild to moderate staining.
Neuraminidase: chondrocytes, clones and matrix showed increased stain. In the OA groups the vessels, vessel matrix and tidemark splits stained.
Fucosidase: normals and ageing unaltered. OA groups increased cell membrane staining zones III and IV.
Fucosidase and neuraminidase: increased cytoplasm and membrane staining. Matrix staining increased but not as intense as neuraminidase alone.
β elimination: diminished chondrocyte staining. Increased matrix staining which dimishes PC>TM>IM.

Figure 4-85 ECA Lectin Staining in Severe OA After Neuraminidase

Very prominent staining of chondrocyte clones. Arrow points to a clone.

Figure 4-86 ECA Lectin Staining in Severe OA After Neuraminidase

Very prominent staining of vessels and splits (not seen in normals). Tidemark staining is absent. Arrow points to a cluster of vessels.

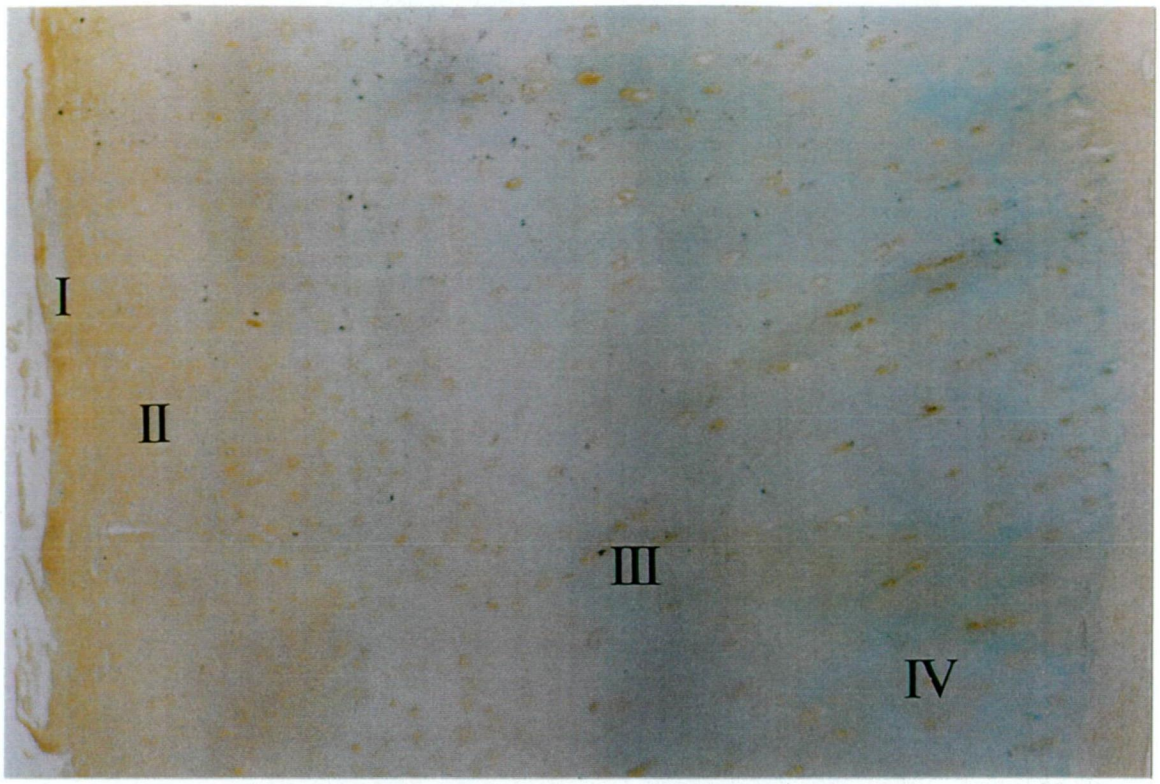


Figure 4-87 ECA Lectin Staining in Early OA After Fucosidase

Increase in intensity and number of chondroctyes staining.

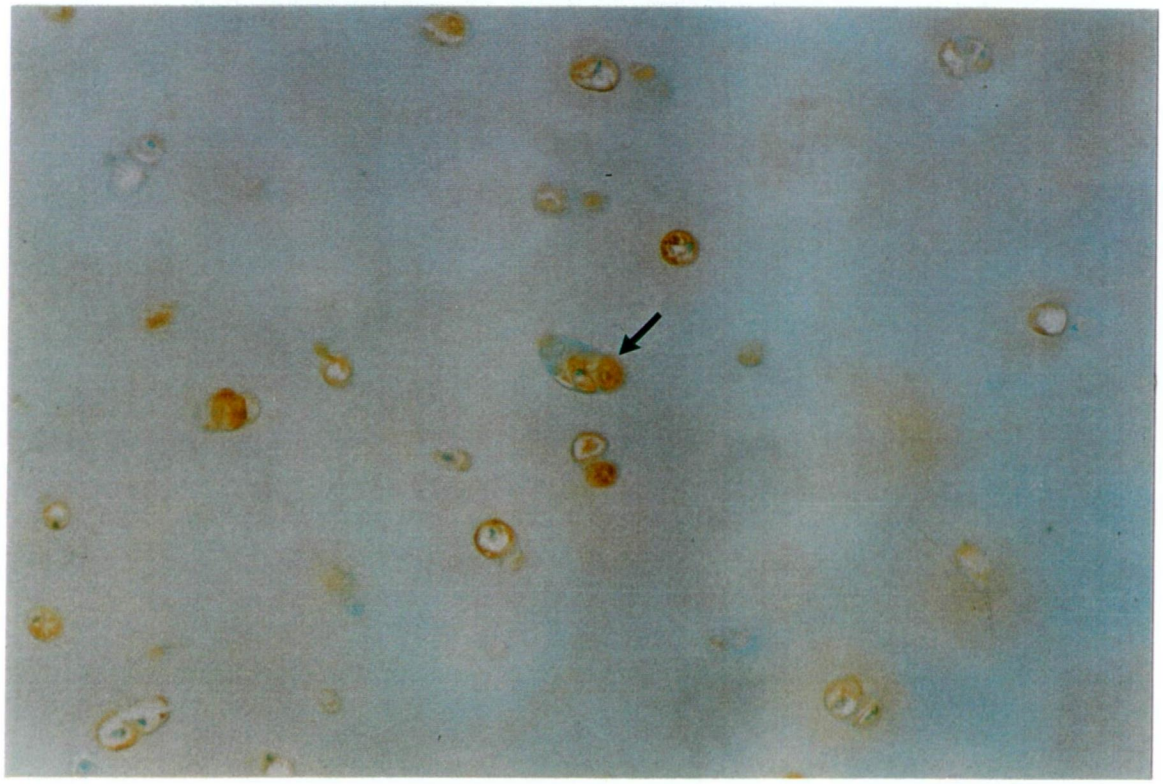


Figure 4-88 ECA Lectin Staining After Neuraminidase + Fucosidase

Very intense staining of chondrocytes. Arrow points to chondrocytes.

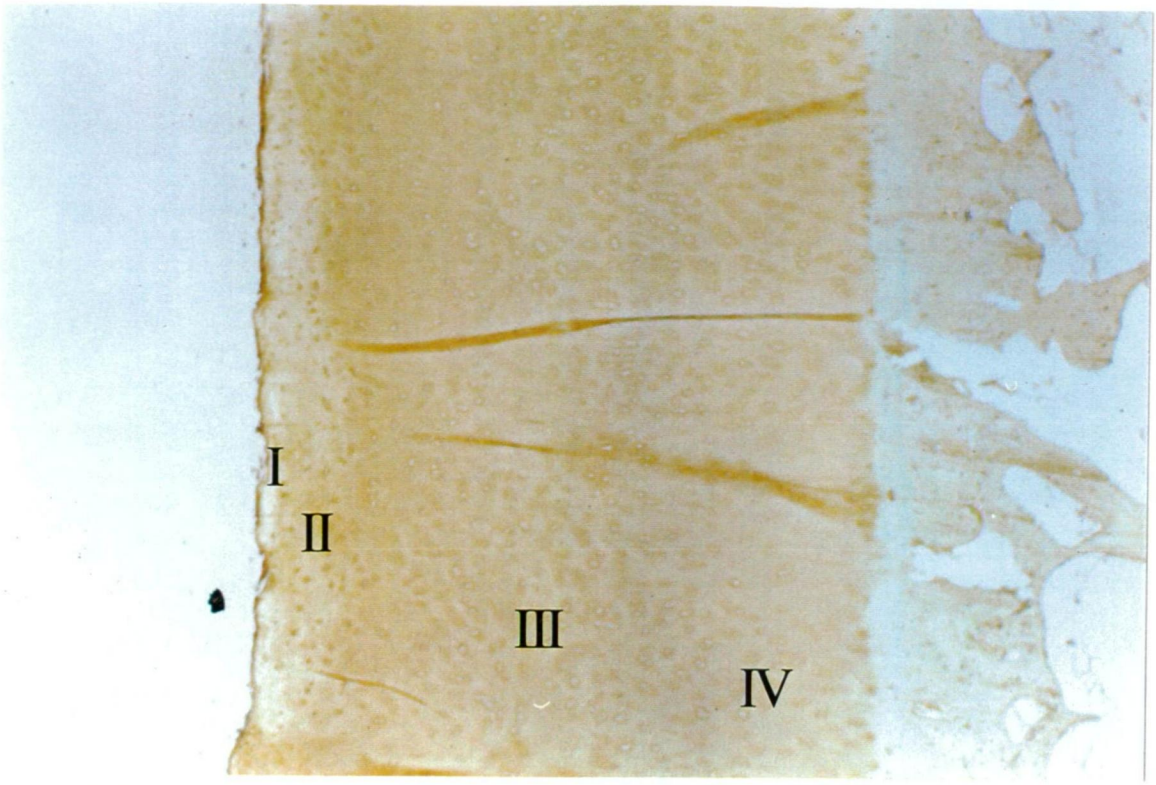


Figure 4-89 ECA Lectin Staining in Early OA After β Elimination

With β elimination there is very marked increase in matrix staining.

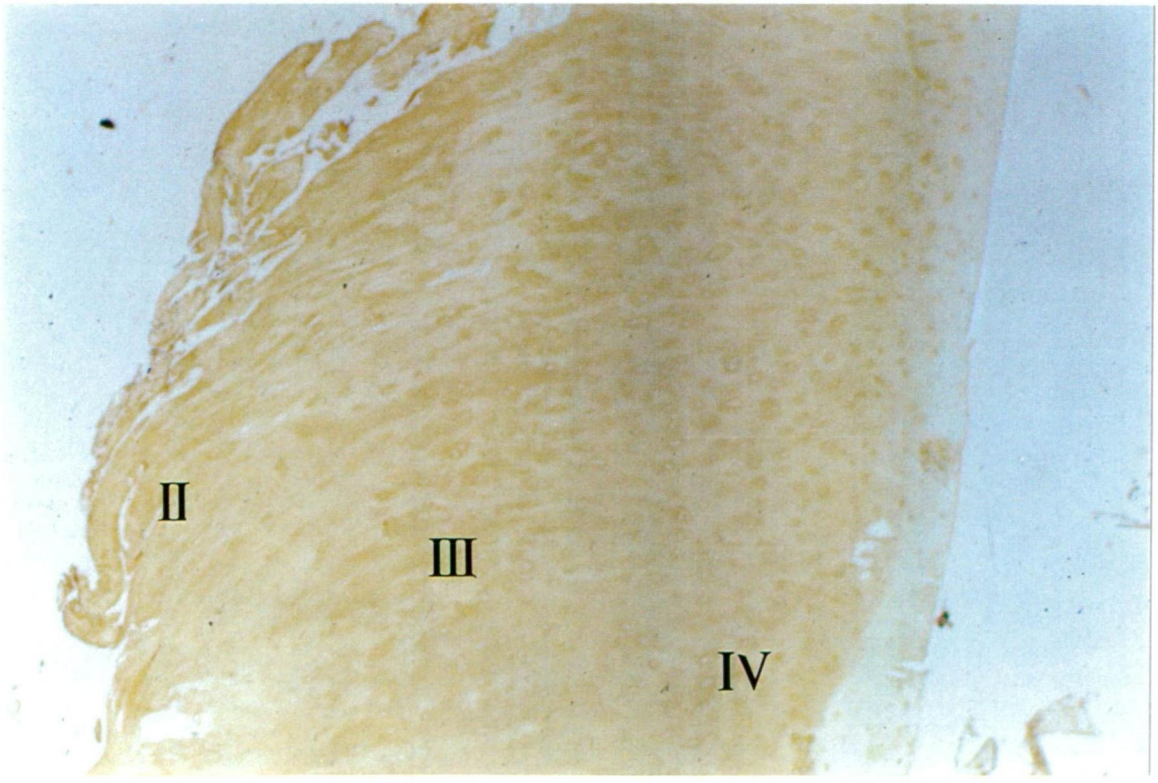


Figure 4-90 ECA Lectin Staining in Severe OA After β Elimination

With β elimination marked increase in matrix staining is carried across all grades.

4.7.4.3 CTA

This lectin does not have a true monosaccharide inhibitor. It appears to have an extended binding site for the disaccharide Gal β 1,4 Glc NAC β 1-, for which is has much higher affinity for the β 1,3 linkage analogue for the disaccharide.

SURFACE: surface staining is absent.

CHONDROCYTE: zone I and II staining was absent. There was mild to moderate cytoplasm stains in zones III and IV in all cases in 20-40% of cells. No cell membrane or peg chondrocytes stained. There was moderate staining of cytoplasm in up to 40% of clones but none in membranes.

MATRIX: matrix staining was totally absent in all groups.

CHONDRO-OSSEOUS JUNCTION: there was no staining of the tidemark, vessels, vessel matrix or splits.

SUBCHONDRAL BONE: osteocytes did not stain. The bone matrix staining was absent in normals, mild in the ageing and early OA groups, and moderate in the moderate severe OA groups.

NEW CARTILAGE: where present there was mild to moderate cytoplasm staining in a small percentage of cases. No membrane or matrix staining was seen.

ENZYMATIC DEGRADATION:

Neuraminidase: there was a dramatic increase in membrane and cytoplasm staining. Matrix stains in all groups and zones with intensity falling off across regions PC>TM>IM.

Fucosidase: there was a very marked increase in chondrocyte staining particularly of the membranes. The matrix showed an increase in staining but not as dramatic as with neuraminidase, this was most pronounced in zones I and II, and in zones III and IV in the pericellular region (PC>>TM>IM. Osteocytes showed some staining.

A summary of the key features is provided in Table 4-16.

Examples of the staining patterns are seen in Figs 4-91 to 4-94.

Table 4-16 Summary of CTA lectin histochemistry Group 4: β - Galactose

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY	PC/TM	IM	TD	MA	OS
0 NORM	0	0	0	0	0/0	0/0	0	2/0	0/0	0	1/0	0/0	0	0	0	0
1 EARLY	0	0	0	0	0/0	0/0	0	2/0	0/0	0	1/0	0/0	0	0	1	0
2 AGED	0	0	0	0	0/0	0/0	0	2/0	0/0	0	1/0	0/0	0	0	1	0
3 MOD	0	0	0	0	0/0	0/0	0	2/0	0/0	0	1/0	0/0	0	0	2	0
4 SEVER	0	0	0	0	0/0	0/0	0	2/0	0/0	0	1/0	0/0	0	0	2	0

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: the only staining was in the cell cytoplasm in all groups in zones III and IV.

Matrix: this does not stain.

Tidemark and osteocytes do not stain. Bone matrix stains with increasing intensity through the groups.

Neuraminidase: dramatic increase in membrane and cytoplasm staining. Matrix stains all groups PC>TM>IM.

Fucosidase: marked chondroctye staining membranes > cytoplasm. Marked staining was increased but not as much as with neuraminidase , PC>TM>IM.

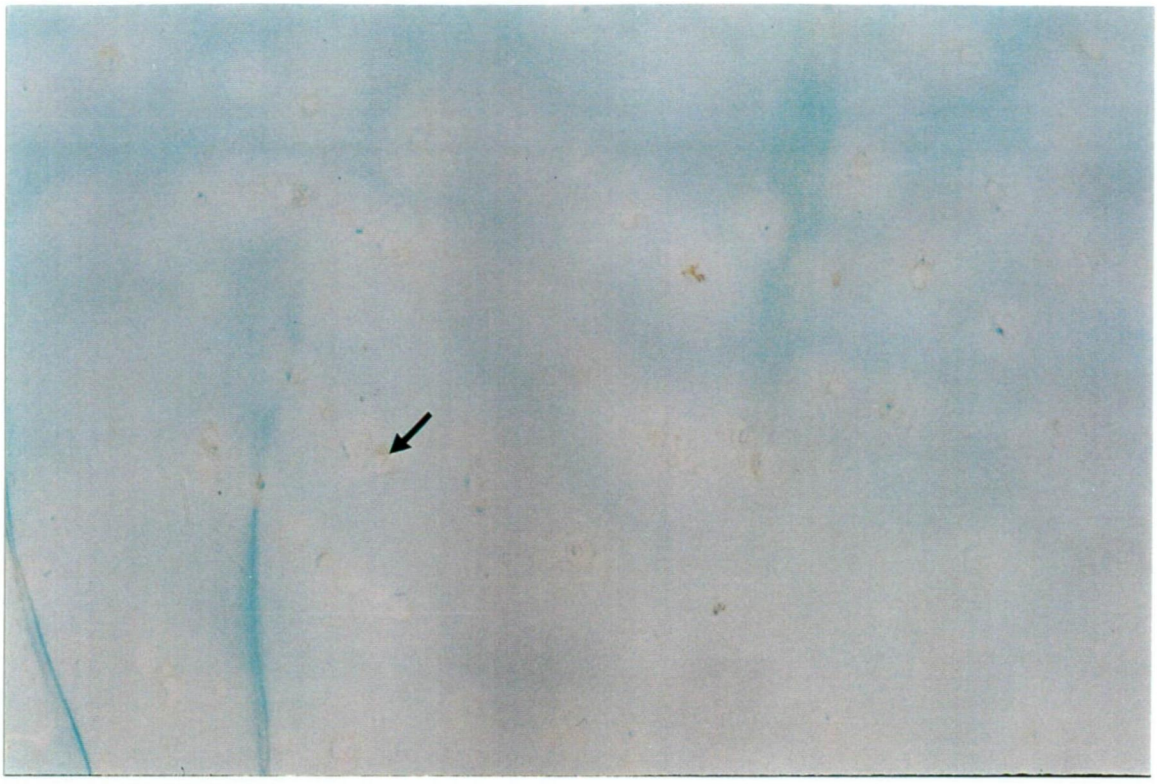


Figure 4-91 CTA Lectin Staining in Early OA Cartilage

Very weak staining of some chondrocytes but otherwise negative. Arrow points to chondrocytes.

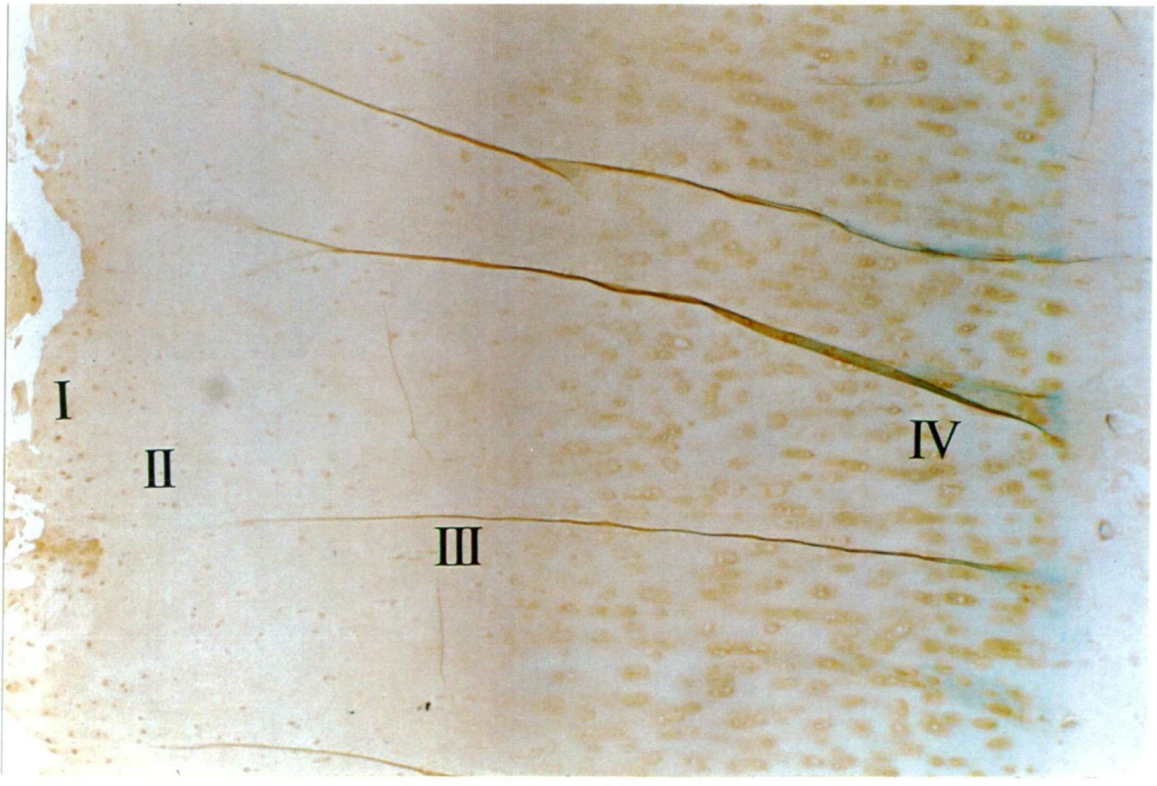


Figure 4-92 CTA Lectin Staining in Early OA Cartilage with Neuraminidase

Dramatic increase in chondrocytes and matrix (PC>TM>IM) staining.

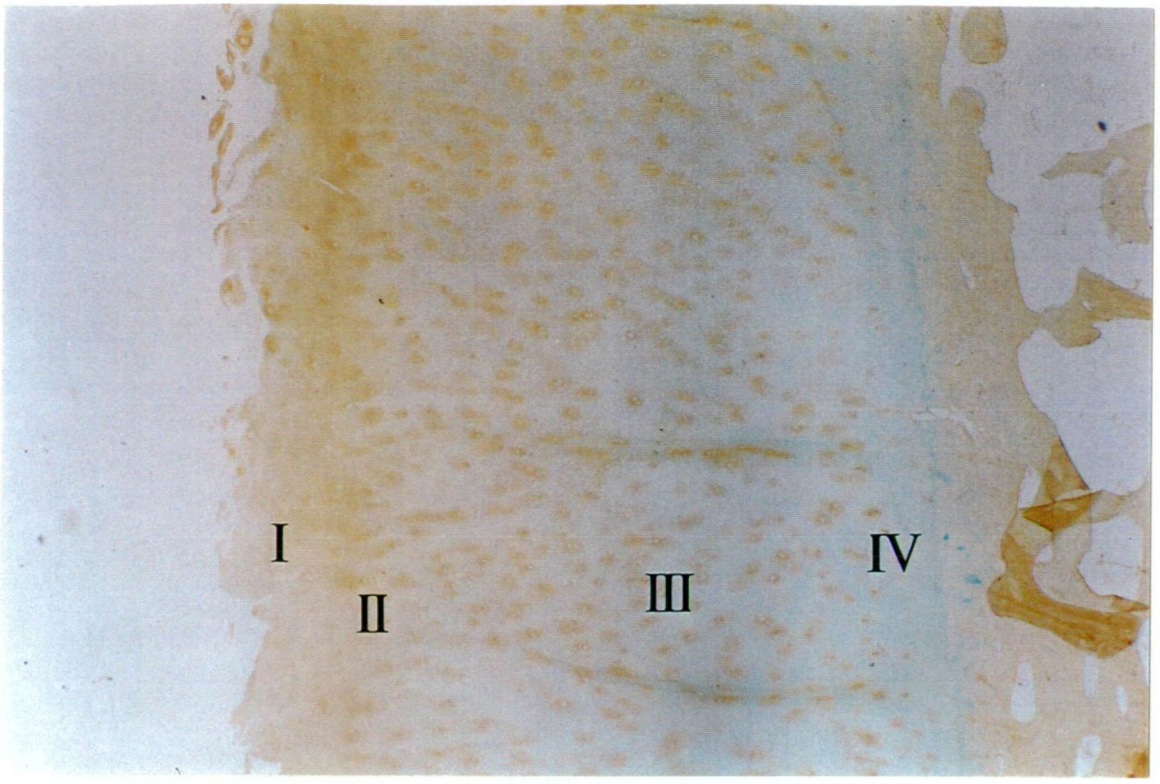


Figure 4-93 CTA Lectin Staining in Moderate OA with Fucosidase

Marked increase in chondrocyte staining. Matrix staining is also increased more so in zones I and II than III and IV; this increase is not as pronounced as with neuraminidase.

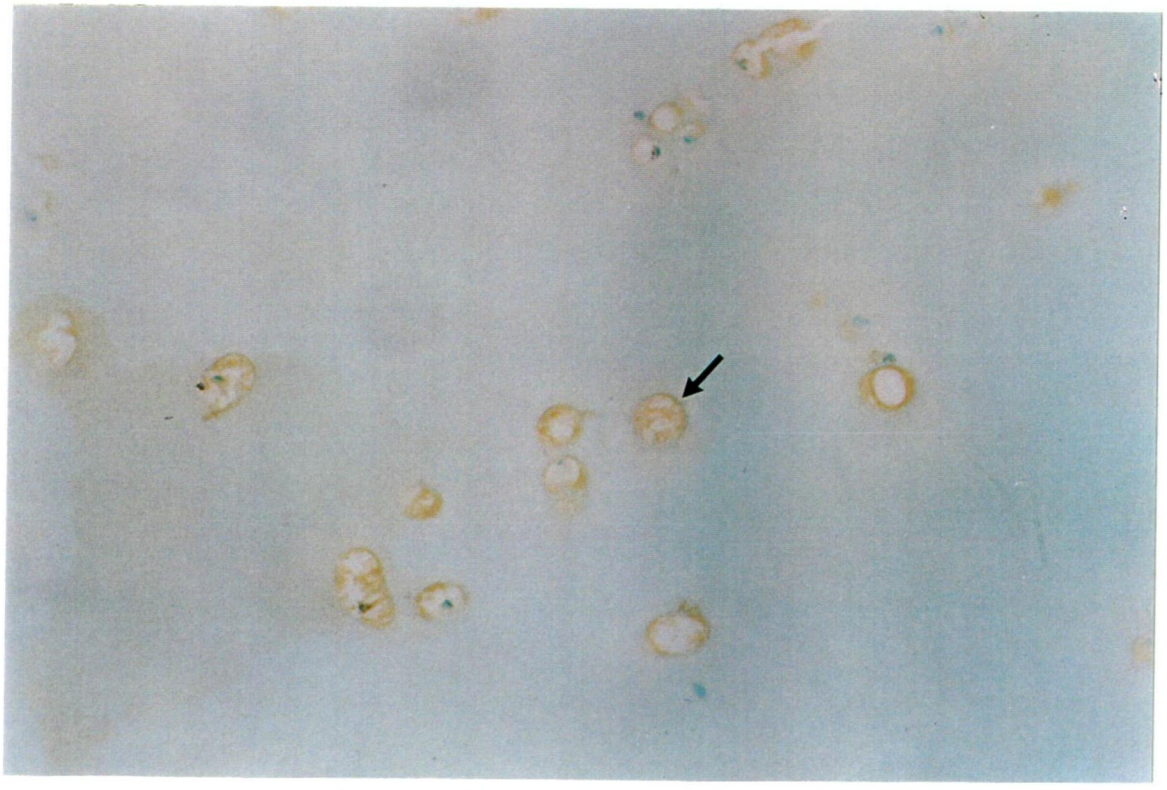


Figure 4-94 CTA Lectin Staining in Zone IV in Moderate OA with Fucosidase

Marked increase in chondrocyte staining. Arrow points to chondrocytes.

4.7.5 GROUP 5 – N – acetyl galactosamine

The lectins in this include DBA, VVA B4, WFA and HPA. DBA also has an affinity for Group 6 lectins.

4.7.5.1 DBA

SURFACE: surface staining was moderate to strong in normals, ageing and early OA groups, and patchy in the mild and severe OA groups.

CHONDROCYTES: no staining of the cell membrane was seen in any of the zones or pegs. Moderate cytoplasm staining was seen in up to 40% of cells in all groups in all zones. Clone cytoplasm and membrane staining was seen only in severe OA with between 40-60% of cells staining.

MATRIX: in the normals there was moderate matrix staining in zone I, mild in of all regions in zone II, and absent in zones III and IV. In the ageing and early OA group the intensity of staining in zones I and II increased with no difference in regions, now very mild zone III staining was noted in some cases. This pattern was continued into the moderate to severe OA groups now with some zone IV staining. There was no staining of zone V, pegs or clones.

CHONDRO-OSSEOUS JUNCTION: all parameters negative.

SUBCHONDRAL BONE: osteocytes negative and bone matrix moderate staining.

NEW CARTILAGE : did not stain.

ENZYMATIC DEGRADATION:

Neuraminidase: increase in chondrocyte staining in all zones and groups, membrane now stain. There was a marked increase in staining in zones I and II, in zone III (PC>TM>IM) and zone IV, this was pericellular only.

β elimination: increase in membrane and cytoplasm staining in all groups and zones most pronounced in zone IV and an increase in the matrix staining – across all groups and zones (PC>>TM>IM).

A summary of the key features is provided in Table 4-17.

Examples of the staining patterns are seen in Figs 4-95 and 4-96.

Table 4-17 Summary of DBA lectin histochemistry Group 5: N-acetyl galactosamine, Group 6 Fucose

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	3	2	0	2	2/0	1/1	1	2/0	0/0	0	2/0	0/0	0	0	2	0
1 EARLY	3	2	0	2	2/0	2/2	2	2/0	1/1	0	2/0	0/0	0	0	2	0
2 AGED	3	2	0	0	2/0	2/2	2	2/0	1/1	0	2/0	0/0	0	0	2	0
3 MOD	2	2	0	2	2/0	2/2	2	2/0	1/1	0	2/0	0/0	0	0	2	0
4 SEVER	2	2	0	2	2/0	2/2	2	2/0	1/1	1	2/0	1/1	0	0	2	0

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was moderate cytoplasm staining in all cases in all zones.

Matrix: the matrix staining was moderate in zones I and II in all groups, absent in zones III and IV in the normals and mild and extending into zone IV as the severity of OA increase.

Tidemark and osteocytes do not stain. Bone matrix showed moderate staining.

Neuraminidase: chondrocyte CY staining was increased and CM now stains. Marked increase in matrix staining.

β elimination: overall increase in matrix and chondrocyte staining.

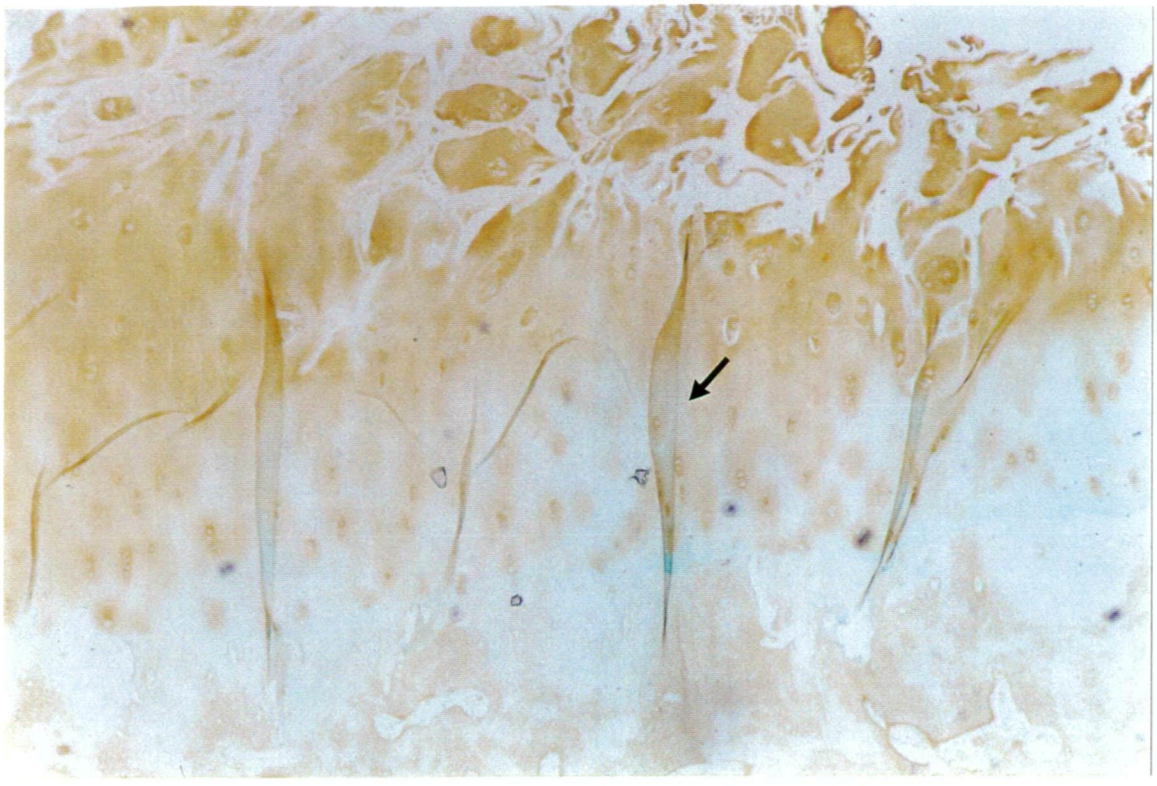


Figure 4-95 DBA Lectin Staining in Severe OA with Neuraminidase

Increased matrix and chondrocyte staining and artifactual creasing of tissue section (Arrow).

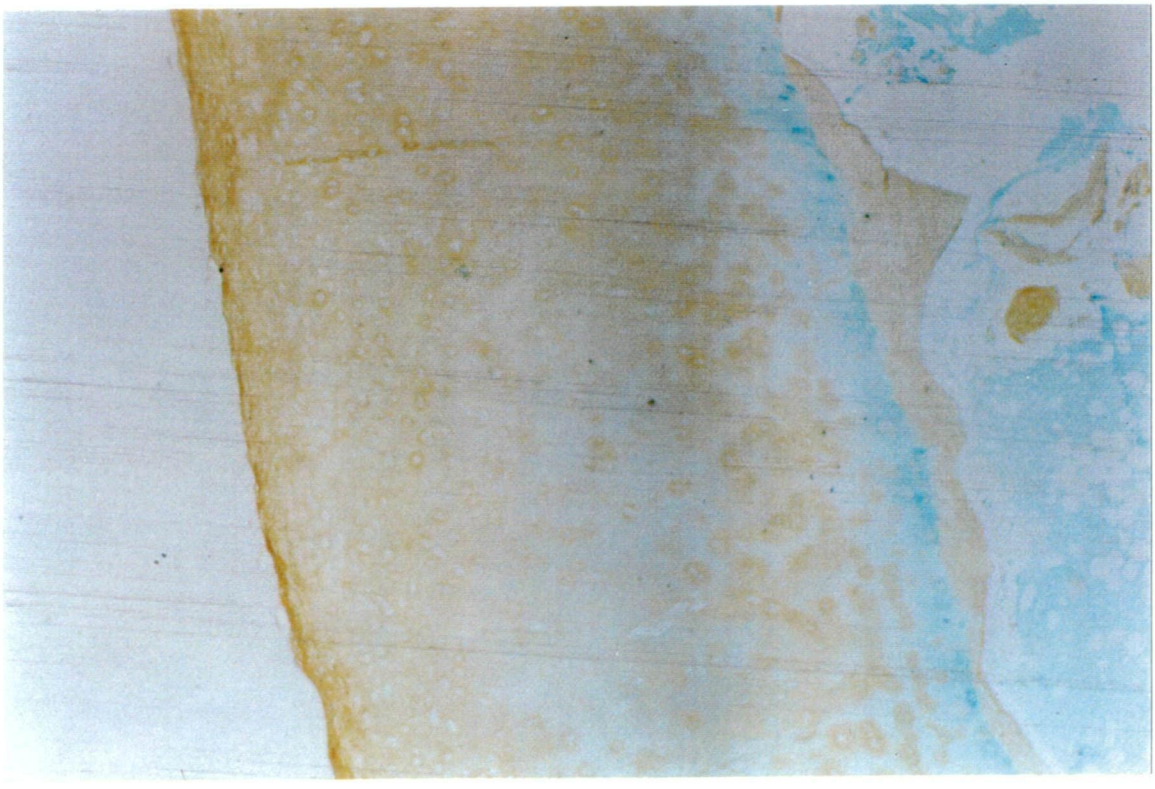


Figure 4-96 DBA Lectin Staining in Normals with β Elimination

Shows increased matrix (PC>TM>IM) and chondrocyte staining. Tidemark and osteocytes do not stain, bone matrix does.

4.7.5.2 VVA B4

SURFACE: moderate in normals and mild and patchy in other groups.

CHONDROCYTES: there was no cell membrane staining in the first four groups. In the severe group a few cases showed mild cell membrane staining in some cells. In the normals there was an absence of cytoplasm staining in zone I and in some cases mild staining in zones II-IV (III greatest staining) in up to 40% of cells. In the ageing and OA groups, zone I and II cytoplasm staining was virtually absent with mild to moderate cytoplasm staining of less than 20% of cells noted in zones III-IV. Some staining of the cytoplasm was seen in the pegs in the first three groups and was absent in the two more severe groups. Clones did not stain.

MATRIX: in the normal group there was mild to moderate matrical staining of zone I, mild staining in all regions of zone II and mild pericellular staining only in zones III and IV. In the ageing, and early OA groups there was a gradual loss of staining in zones I-III with virtual absence in zone IV. In the moderate OA group there was a slight increase in pericellular staining in zones III and IV. In the severe OA group staining was almost completely absent. Zone V matrix was negative apart from being patchy in severe OA group. Clones did not stain.

CHONDRO-OSSEOUS JUNCTION: there was no staining of the tidemark, vessels, vessel matrix or splits.

SUBCHONDRAL BONE: no staining of osteocytes or matrix.

NEW CARTILAGE: there was no staining of any parameters of new cartilage.

ENZYMATIC DEGRADATION:

Neuraminidase: Increased staining of cytoplasm in all groups and zones and also of matrix (II/III >IV). Granular staining was seen in zone V.

β elimination: there was increased cytoplasm and membrane staining across all zones and groups. Increased matrix staining was seen in all zones and groups; this was more pronounced in the upper zones and pericellular region (PC>TM>IM).

A summary of the key features is provided in Table 4-18.

An example of the pattern of staining is seen in Figure 4-97.

Table 4-18 Summary of VVA B4 Lectin Histochemistry Group 5: N–acetyl galactosamine

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	0	0	1	1/0	1/1	1	1/0	1/1	1	1/0	1/0	0	0	0	0
1 EARLY	1	0	0	1	0/0	1/1	1	1/0	1/1	0	1/0	0/0	0	0	0	0
2 AGED	1	0	0	1	0/0	1/1	1	1/0	1/1	0	1/0	0/0	0	0	0	0
3 MOD	1	0	0	1	0/0	1/1	1	1/0	1/1	0	1/0	1/0	0	0	0	0
4 SEVER	1	0	0	1	0/1	0/0	0	1/1	0/0	0	1/1	0/0	0	0	0	0

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocyte: cytoplasm staining is absent in zones I and II and was mild in zones III and IV. Membrane staining was only seen in the severe group in zones III and IV.

Matrix: mild zone I and II staining was present across all regions and in all groups. In zones III and IV staining decreased across regions PC>TM>>IM and with increasing disease.

Tidemark, osteocyte and matrix staining was absent.

Neuraminidase: increased cytoplasm and matrix staining in all zones and groups.

β elimination: increased cytoplasm, membrane and matrix staining (PC>TM>IM) in all groups and zones.

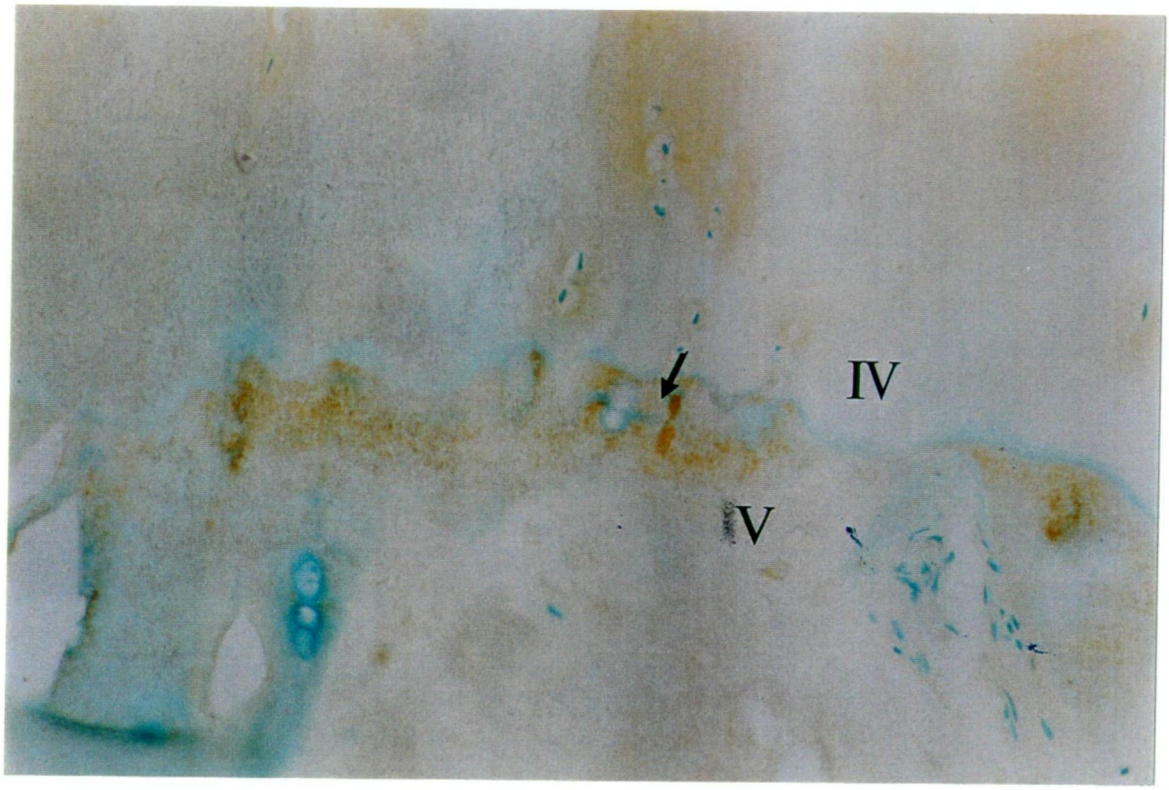


Figure 4-97 VVA B4 Lectin Staining in Moderate OA at the Tidemark with Neuraminidase

Very prominent granular cytoplasm adjacent to the tidemark in zone V. Arrow points to this region.

4.7.5.3 WFA

SURFACE: moderate too strong in all groups.

CHONDROCYTES: in the normals there was moderate cytoplasm and membrane staining in all zones, in zones I and II 20-40% of cells stain and in zones III and IV 80%. In the ageing group there was a decrease in the intensity and percentage of cells staining in zones I and II and an increase in zones III and IV. In the early OA group there was no staining in zones I and II and moderate in zones III and IV. In the moderate and severe group there was moderate staining of 60-80% of cells in zones I-IV (slightly less cells in zones III and IV). The staining of the pegs was variable with mild cytoplasm staining of 40% of cells and moderate membrane staining in all cases. Staining of clones in ageing and early OA was absent whereas in the moderate group this was moderate in 20-40% of cells and in 80% in the severe group.

MATRIX: there was moderate to strong matrix staining in zones I – IV (PC>>TM>IM). Zone V, peg, periclonal and interclonal matrix staining was mild.

CHONDRO-OSSEOUS JUNCTION: tidemark did not stain. Staining of vessels, vessel matrix and splits was absent in the normal group and present with increasing intensity through the other groups.

SUBCHONDRAL BONE: strong staining of osteocytes and bone matrix.

NEW CARTILAGE: cells moderate, matrix absent.

ENZYMATIC DEGRADATION:

Neuraminidase: uniform increase in the matrix staining, more pronounced in the upper zones (PC>TM>IM) . The chondrocytes showed less staining.

β elimination: there was a uniform increase in matrix staining, particularly in the upper zones and pericellular region. There was little change in cell staining in zones I - III but marked cytoplasm and membrane staining in zone IV. Osteocytes show a striking increase in staining.

A summary of the key features is provided in Table 4-19.

An example of the staining pattern is seen in Figure 4-98.

Table 4-19 Summary of WFA Lectin Histochemistry Group 5: N-acetyl galactosamine

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	2	2	3	2/2	3/3	2	2/2	2/1	1	2/2	2/1	1	0	3	3
1 EARLY	2	0	0	3	0/0	3/3	2	2/2	2/1	1	2/2	2/1	1	0	3	3
2 AGED	3	1	1	3	1/1	3/3	2	3/3	2/1	1	3/3	2/1	1	0	3	3
3 MOD	3	2	2	3	2/2	3/3	2	2/2	2/1	1	2/2	2/1	1	0	3	3
4 SEVER	3	2	2	3	2/2	3/3	2	2/2	2/1	1	2/2	2/1	1	0	3	3

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: the cytoplasm and membranes showed moderate to strong staining in all groups and zones apart from zones I and II in the normals when it was mild or absent.

Matrix: matrix staining was strong in all groups in zones I and II and the PC region and mild in the TM and IM regions.

Tidemark: did not stain; osteocytes and bone matrix were strong.

Neuraminidase: there was a uniform increase in the matrix staining; this was more pronounced in PC than TM or IM. Chondrocytes probably show a decrease.

β elimination:uniform increase in matrix staining PC>TM>IM. Chondroctyes stain more in zone IV; osteocytes stain a lot more.

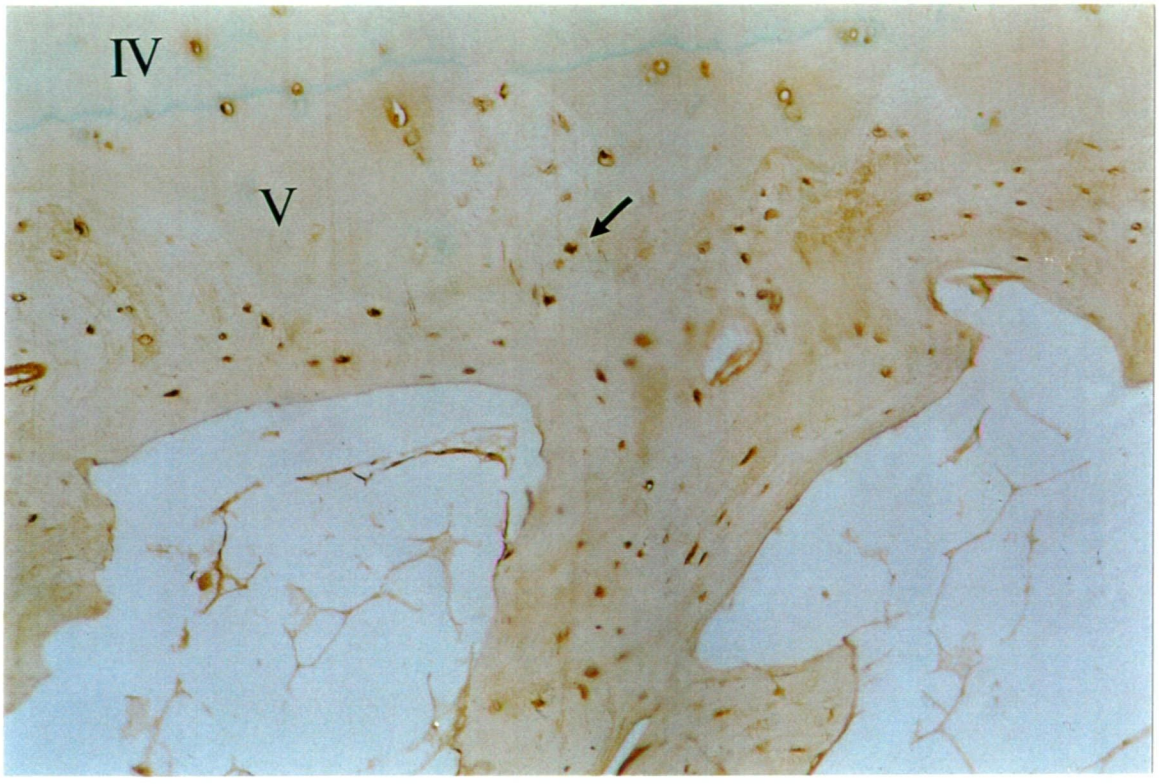


Figure 4-98 WFA Lectin Staining in Subchondral Bone with β Elimination

Very prominent osteocyte staining (arrow) but none of tidemark.

4.7.5.4 HPA

SURFACE: mild to moderate surface staining was present in all groups.

CHONDROCYTES: in the normals staining was absent in zones I and II and was mild in the cytoplasm of 80% of cells. In zones III and IV membrane was negative. In the ageing group there was moderate membrane staining in zones I and II in 20-80% of cells, mild cytoplasm in 30%-50% of cells in zone III and none in zone IV. In the OA groups in zones I and II there was mild cytoplasmic staining of <20% of cells in <20% of cases whereas in zone III there was mild staining in all cases in 60% of cells. There was no membrane or peg chondrocyte staining. Clones in all groups showed moderate cytoplasm staining in between 60-100% of cells, whereas mild membrane staining was seen in the severe group only.

MATRIX: there was mild matrix staining in the normal group in zones I and II, patchy zone III staining and none in zone IV. No difference was seen in the regions. In the ageing and OA groups very mild staining was seen in zone I, occasional in II and III and none in IV. In severe groups loss extended to zone III. Zone V staining was absent in the normal and mild OA groups and moderate in the others. Clonal matrix staining was seen in mild OA only. Peg matrix did not stain.

CHONDRO-OSSEOUS JUNCTION: Tidemark was negative. Vessels, vessel matrix and the splits showed patchy staining in the normal and OA's and was absent in the ageing group.

SUBCHONDRAL BONE: no staining of osteocytes and patchy in matrix.

NEW CARTILAGE: where present there was mild to moderate cytoplasmic staining in up to 60% of cells. Cell membrane and matrix staining was absent.

ENZYMATIC DEGRADATION:

Neuraminidase: in the normal group there was a moderate increase in matrix staining in all zones, not sustained in the severe groups. Chondrocyte staining was increased in the normals and to a lesser extent in the other groups most in zones III and IV.

Most striking was the osteocyte staining with clear dendritic processes abutting onto the marrow spaces in zone V cartilage. Coarse granular material was seen present adjacent to pegs and tidemark staining was now present.

β elimination: there was moderate increase in matrix staining, most marked in zones I, II and the pericellular region. Chondrocyte staining was either absent or decreased.

A summary of the key features is provided in Table 4-20.
Examples of the staining patterns are seen in Figs 4-99 to 4-102.

Table 4-20 Summary of HPA Lectin Histochemistry Group 5: N-acetyl galactosamine

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	0	0	1	0/0	1/1	1	2/0	1/0	0	2/0	0/0	0	0	0	0
1 EARLY	2	1	1	1	1/0		1	1/0	1/0	0	1/0		0	0	0	0
2 AGED	2	1	0	1	1/1	1/1	1	2/0	1/0	0	0/0	0/0	0	0	0	0
3 MOD	2	1	0	1	1/0	1/1	1	1/0	0/0	0	1/0	0/0	0	0	0	0
4 SEVER	2	1	0	1	1/0		1	1/0	0/0	0	1/0	0/0	0	0	0	0

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: there was mild cytoplasmic staining in most groups in all zones; membrane staining was virtually absent.
Matrix: this was mild in all groups in zone I and II, only in the PC region normals, ageing and EOA's zone III and absent in zone IV.
Overall the tidemark, osteocytes and bone matrix did not stain.
Neuraminidase: mild increase in matrix staining was seen. The chondrocyte cell membrane and cytoplasm now stains. There was very striking staining of osteocyte and tidemark staining.
β elimination: chondrocyte staining was overall reduced or absent. Matrix staining increased especially in zones I, II and the pericellular region.

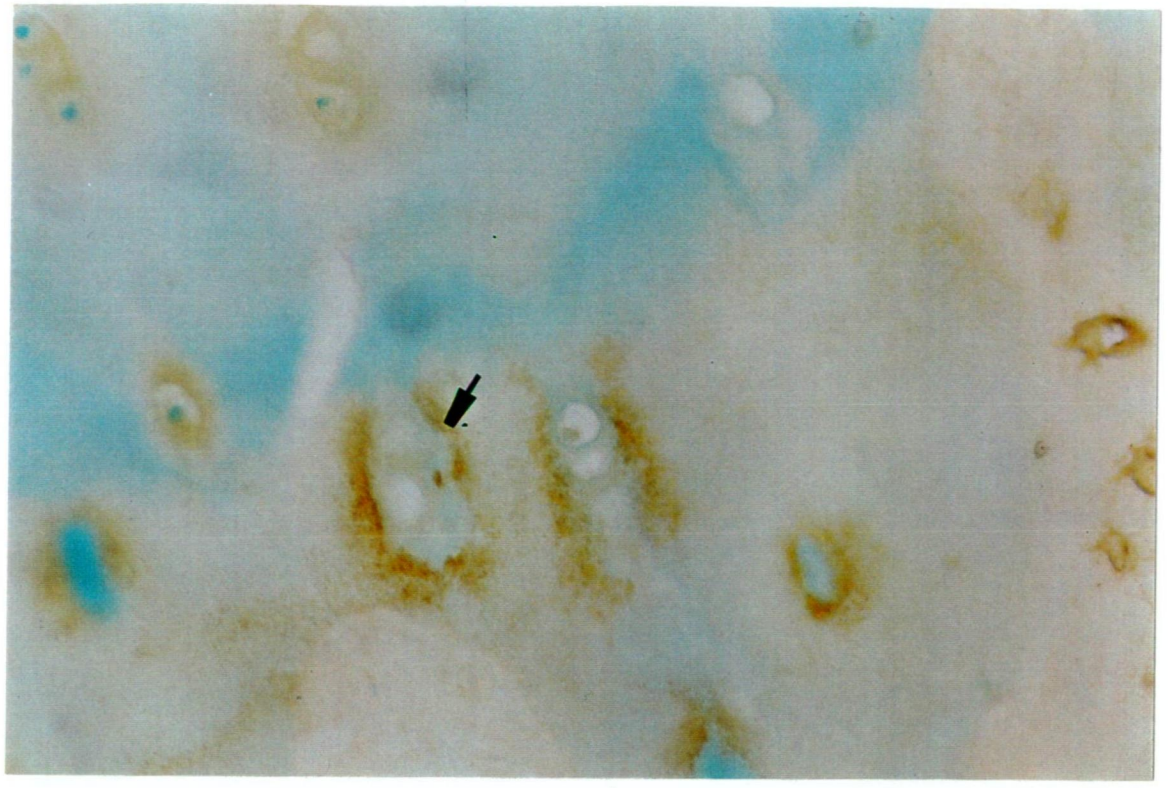


Figure 4-99 HPA Lectin Staining at the Tidemark with Neuraminidase

Very prominent granular deposits in zone V adjacent to the uncalcified cartilage pegs (Arrow).

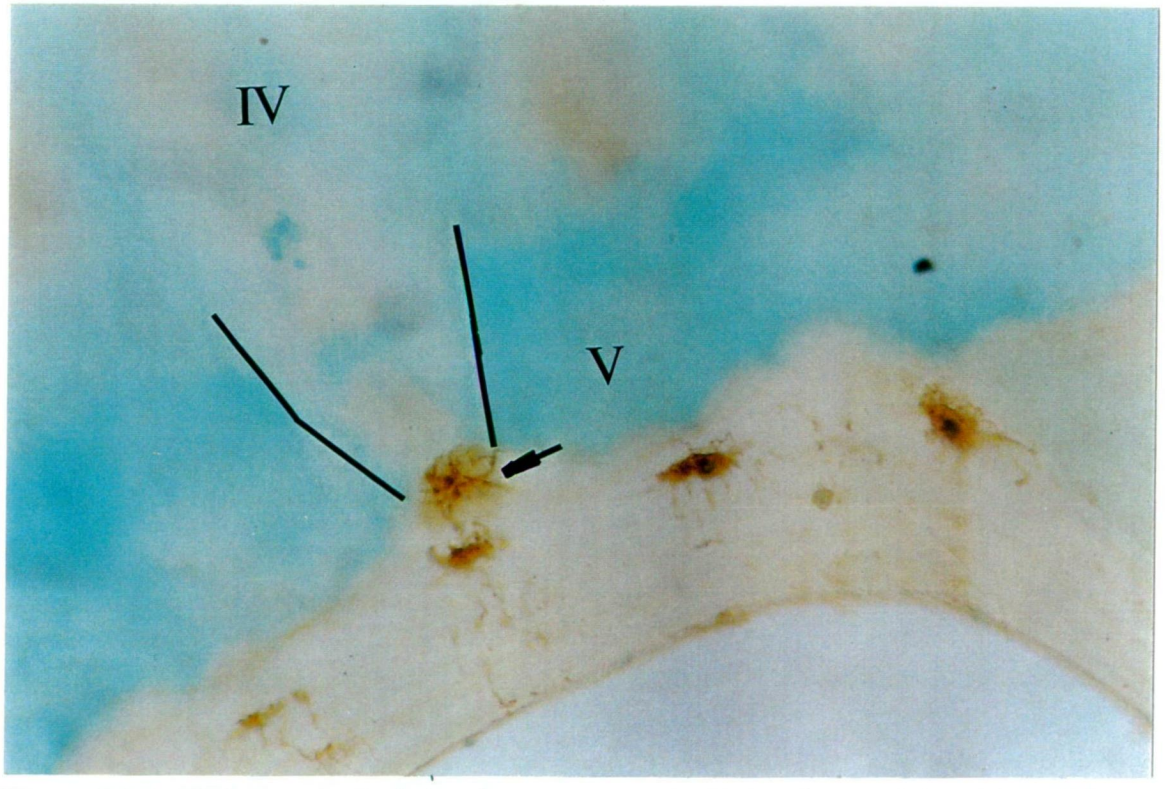


Figure 4-100 HPA Lectin Staining (Normals) at the Osteochondral Junction with Neuraminidase

Prominent osteocytes with dendritic processes abutting onto pale staining uncalcified cartilage pegs. Lines highlight the peg and the arrow points to a chondrocyte.

Figure 4-101 HPA Lectin Staining (Normals) at Osteochondral Junction with Neuraminidase

Prominent osteocytes with dendritic processes (arrow) seen abutting onto the marrow space.

Figure 4-102 HPA Lectin Staining (Normals) with β Elimination

Prominent increase in pericellular staining.

4.7.6 GROUP 6 – FUCOSE

The only lectin included in this group was LTA.

4.7.6.1 LTA

SURFACE: mild to moderate staining was seen in all groups.

CHONDROCYTES: in normals in zone I cytoplasm and membrane staining was absent in all cases. In the normal group in zone II-IV there was very mild cytoplasm and membrane staining in up to 60% of cells in a few cases. In the ageing and early OA groups the staining pattern was absent in zone I, moderate in the cytoplasm in a small percentage of cells and absent in the membrane in zone II and moderate in the cytoplasm and membrane in up to 40% of cells in zones III and IV. In the moderate and severe OA groups there was no zone II staining. Staining was mild to moderate in the cytoplasm and absent in the membrane in up to 60% of cells in zones III and IV. The peg chondrocytes did not stain in normals, in OA groups there was moderate cytoplasm and membrane staining in about 50% of cases in up to 60% of cells. Clones stained in some cases.

MATRIX: there was mild to moderate matrix staining in zone I and all regions of zone II. In zones III and IV there was mild staining (PC>>TM>IM), in thetwo severe OA groups zone IV staining was absent. Zone V matrical staining was present in the first few groups and absent in the severe group. Intraclonal matrix was seen in some cases in the severe group.

CHONDRO-OSSEOUS JUNCTION: no staining of parameters.

SUBCHONDRAL BONE: some osteocytes showed mild staining in the severe group. There was moderate bone matrix staining in all groups.

ENZYMATIC DEGRADATION: ***β elimination***: there was an increase in matrix staining, most marked in the upper zones and PC regions. There was some increase in membrane staining in zones III and IV.

Fucosidase: there is an increase in matrix staining most marked in zones I, II and the PC region; this increase diminished with severity of disease. Membrane and cytoplasm staining was more in all groups particularly in zones III and IV.

A summary of the key features is provided in Table 4-21.

Table 4-21 Summary of LTA Lectin Histochemistry Group 6: β - Galactose

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	0	0	2	1/1	2/2	1	2/2	2/1	0	2/2	2/1	1	0	0	2
1 EARLY	2	0	0	2	2/0	2/2	1	2/2	2/1	0	2/2	2/1	1	0	0	2
2 AGED	2	0	0	2	2/0	2/2	1	2/2	2/1	0	2/2	2/1	1	0	0	2
3 MOD	2	0	0	2	0/0	2/2	1	2/0	2/1	0	1/0	1/0	0	0	0	2
4 SEVER	2	0	0	2	0/0	2/2	1	2/0	2/1	0	1/0	1/0	0	0	1	2

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: zone I, CM and CY was absent; zone 2, CY was mild to moderate in less severe groups and CM was absent; zones III-IV CM and CY was mild to moderate except in severe groups.

Matrix: was moderate in all regions in zones I and II and fell off in intensity across the regions in zones III and IV, PC>TM>IM.

Osteocyte and tidemark did not stain. The matrix showed moderate staining.

β elimination: increased matrical staining especially zones I, II and the pericellular region. Chondrocyte matrix and membrane show increased staining in zones III and IV.

Fucosidase: virtually identical to β elimination.

4.7.7 GROUP 7 – SIALIC ACID

This group of lectins binds to sialic acid and include MAA, SNA and LFA.

4.7.7.1 MAA

SURFACE: mild to moderate staining in all groups.

CHONDROCYTES: in the normal group CY and CM staining was present in all zones I<II<III<IV in 60-100% of cells. In the ageing group in zones I and II staining of CM was lost and that of the CY became weak in 20-40% of cells and zones III and IV were the same as the normals. In the OA group staining of CM and CY was mild in zones I and II in up to 40% of cells and moderate in zones III and IV in up to 60% of cells. Moderate CY staining was present in the peg chondrocytes in all groups, however, CM staining was present in the ageing group only and these features were seen in 20-40% of cells, clones showed moderate to strong staining.

MATRIX: in the normal, moderate and severe OA group in 50% of cases the staining pattern was mild in zones I and II, very mild in zone III and absent in zone IV. With the ageing and early OA groups there was mild to moderate staining of zones I-III in all regions and none in zone IV. There was no zone V or peg matrix staining and was mild in the intra- and periclonal matrix.

CHONDRO-OSSEOUS JUNCTION: the tidemark did not stain. There was patchy staining of vessels and vessel matrix in the normal, ageing and severe groups and a virtual absence of staining in the early and mild OA groups.

SUBCHONDRAL BONE: mild to moderate staining of osteocytes and bone matrix.

NEW CARTILAGE: mild cytoplasmic and membrane staining in 20% of cells with no matrical staining.

ENZYMATIC DEGRADATION: ***Neuraminidase:*** decreased staining in both matrix and chondrocytes was seen.

β elimination: overall increase in matrix stain with no differentiation of regions until the MOA and SOA groups where PC>TM>IM. In the ageing and EOA groups there was a marked increase in CY staining and this was now seen in the CM.

A summary of the key features is provided in Table 4-22.

Examples of the staining patterns are seen in Figs 4-103 to 4-106.

Table 4-22 Summary of MAA Lectin Histochemistry Group 7: Sialic acid

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	1	1	1	1/1	1/1	1	2/2	1/1	0	2/2	0/0	0	0	2	2
1 EARLY	2	1	1		1/1	2/1	1	2/0	2/1	1	2/0	0/0	0	0	2	2
2 AGED	2	1	1	1	1/1	2/1	1	2/2	2/1	1	2/2	0/0	0	0	2	2
3 MOD	1	1	0	1	1/0	1/1	1	2/0	1/1	0	2/0	0/0	0	0	2	2
4 SEVER	1	1	0	1	1/0	1/1	1	2/0	1/1	0	2/0	0/0	0	0	2	2

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondroctyes: mild-moderate CY and CM staining throughout and CM is lost in severe groups.
Matrix: mild – moderate zones I-III, absent zone IV.
Tidemark: absent; osteocytes and bone matrix – moderate.
Neuraminidase: either no change or decrease in intensity in all parameters.
β elimination: overall increase in matrix staining and increased CY and CM particularly in EOA and ageing.

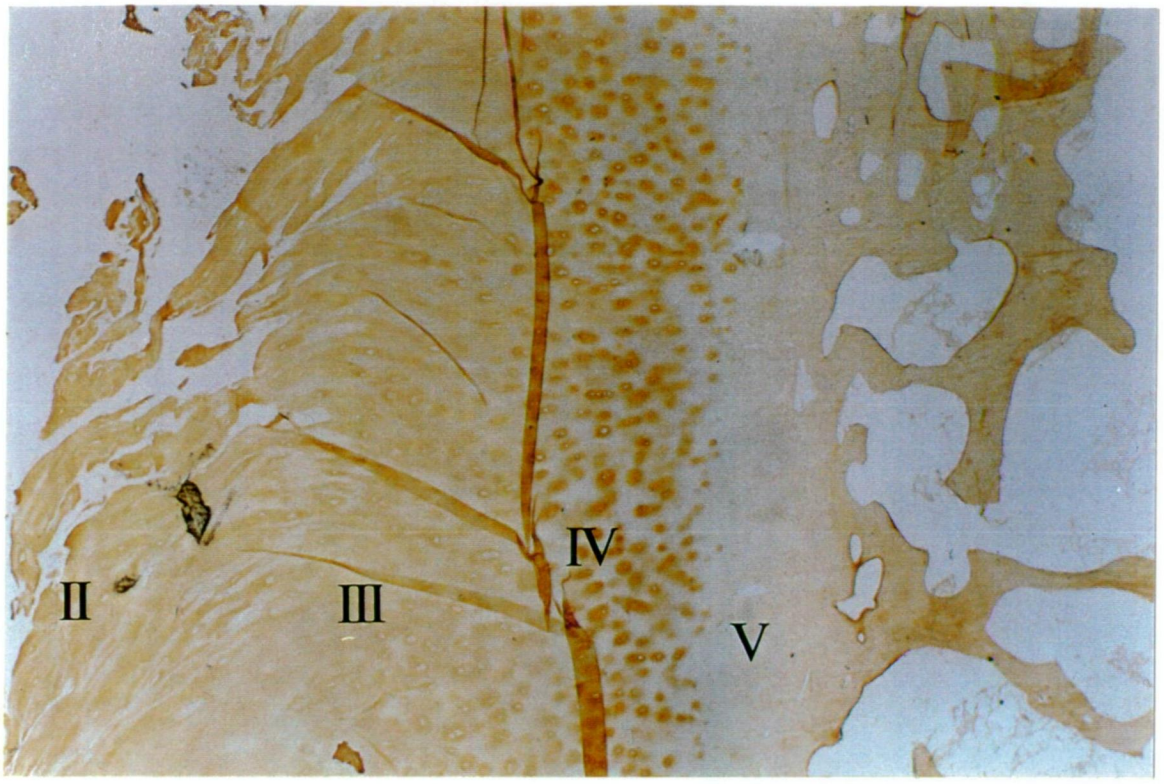


Figure 4-103 LTA Lectin Staining in Severe OA with Fucosidase

Main feature is marked increase in matrix staining in zone I and II and pericellular region.

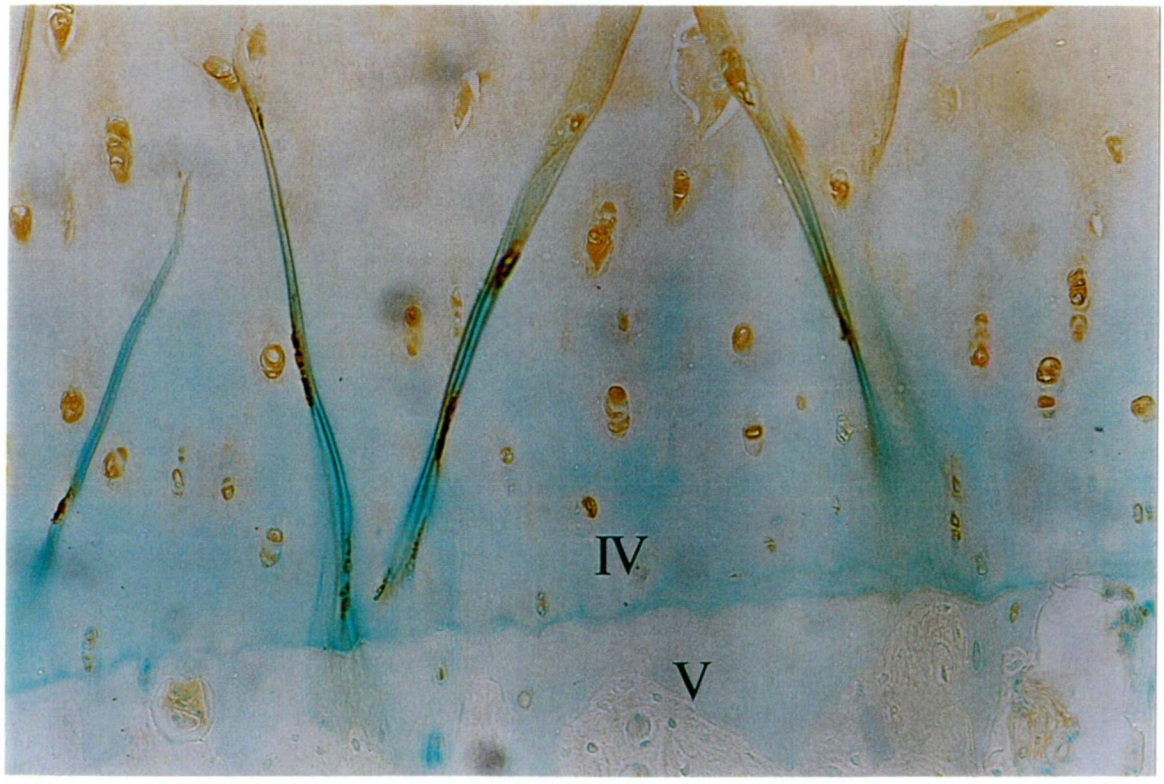


Figure 4-104 MAA Lectin Staining in Ageing

Shows good chondrocyte staining and absence of zone IV matrix and tidemark staining.

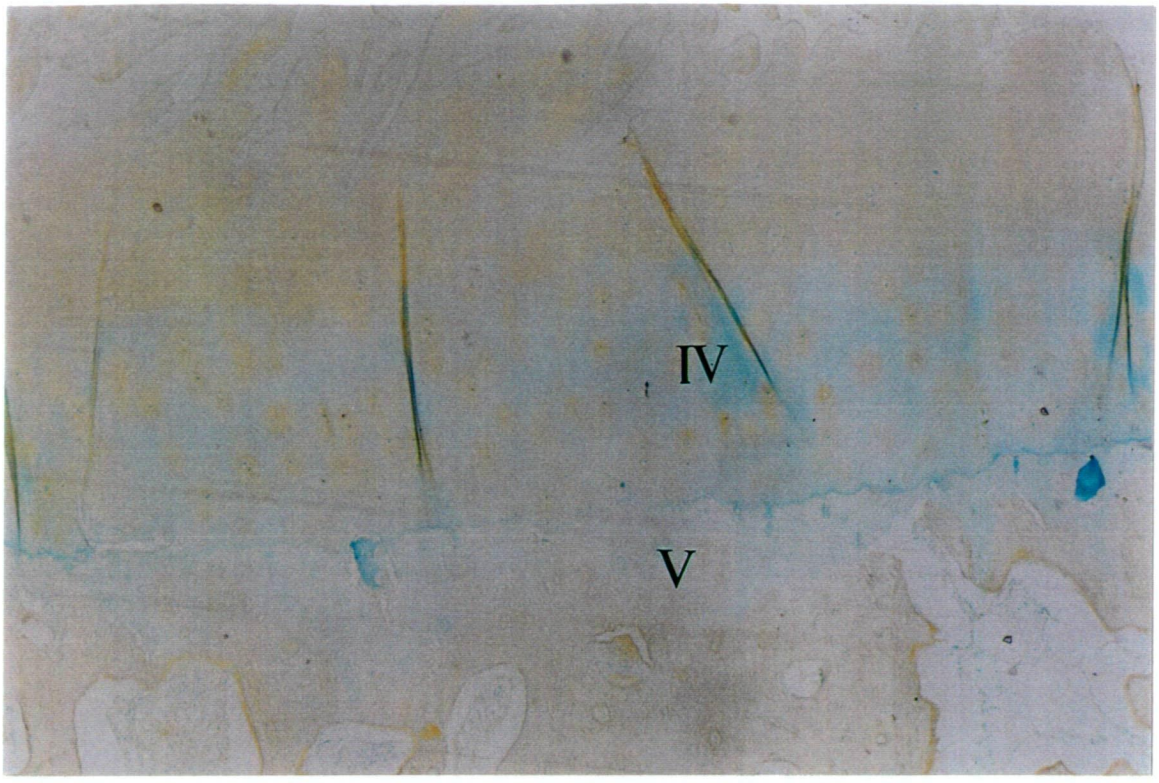


Figure 4-105 MAA Lectin Staining in Severe OA with Neuraminidase

Marked decrease in matrix and chondrocyte staining. Tidemark staining is absent.

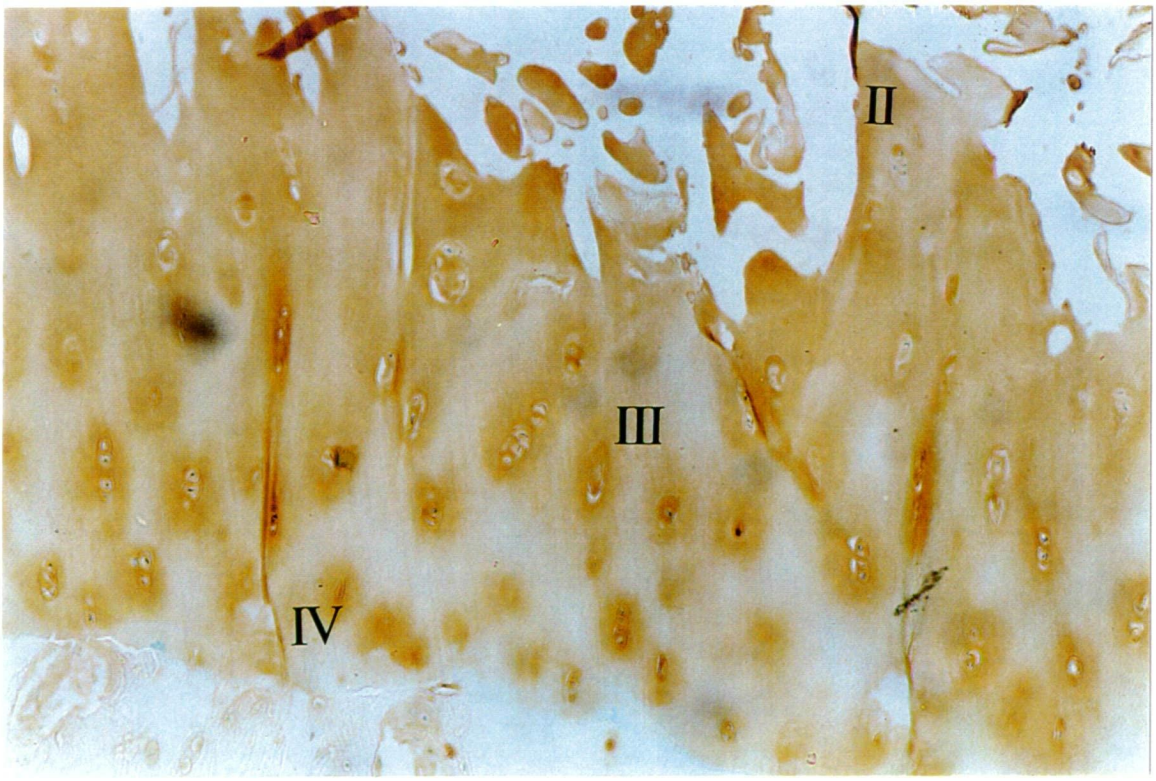


Figure 4-106 MAA Lectin Staining in Severe OA with β Elimination

Marked increase in matrix staining with clear definition between regions particularly in deeper zones.

4.7.7.2 SNA

SURFACE: surface staining was moderate to strong.

CHONDROCYTES: cytoplasm and membrane staining was present in all zones. In the normal groups there was mild to moderate staining in zones I and II and moderate to strong in zones III and IV. In the ageing and osteoarthrotic groups there was a slight increase in intensity across all zones, I>II>III>IV. The staining pattern of peg chondrocyte cytoplasm and membrane was similar. The cytoplasm and membrane of clones showed moderate to strong staining in all cases in all groups.

MATRIX: in the normal group there was mild to moderate granular staining across all zones and regions. In ageing and OA groups there was a slight increase in staining intensity in zones I and II in all regions and a slight loss in zones III and IV, particularly in the more severe groups. There was mild staining of zone V matrix, moderate staining of peg matrix. There was moderate staining of clonal matrices, less intense in the severe group.

CHONDRO-OSSEOUS JUNCTION: the tidemark did not stain. Vessel staining was absent in the normal and ageing groups and mild to moderate in the other groups. Vessel matrix and tidemark split staining was mild to moderate in all groups.

SUBCHONDRAL BONE: moderate staining of osteocytes and matrix.

NEW CARTILAGE: where present the cytoplasm and membranes showed strong staining with a patchy distribution. Matrix showed moderate staining.

ENZYMATIC DEGRADATION: ***Neuraminidase:*** there was a very marked decrease in matrix staining in all groups. Cytoplasm and membrane staining increased.

β elimination: there was a marked uniform increase in matrix staining over all zones and regions, PC>TM>IM. Chondrocytes stain less.

A summary of the key findings is provided in Table 4-23.

Examples of the staining patterns are seen in Figs 4-107 to 4-110.

Table 4-23 Summary of SNA Lectin Histochemistry Group 7: Sialic acid

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	2	2	2	2/2	2/2	2	3/3	2/2	2	3/3	2/2	2	0	1	1
1 EARLY	2	3	3	3	3/3	3/3	3	3/3	2/2	2	3/3	2/2	2	0	1	1
2 AGED	2	3	3	3	3/3	3/3	3	3/3	2/2	2	3/3	2/2	2	0	1	1
3 MOD	2	3	3	2	3/3	2/2	2	3/3	2/1	1	3/3	2/1	1	0	1	1
4 SEVER	2	3	3	2	3/3	2/2	2	3/3	1/1	1	3/3	2/1	1	0	1	1

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: moderate to strong CM and CY in all zones in all groups.
Matrix: is mild to moderate in all groups and zones, there is some decrease in intensity in the MOA and SOA groups and in zones III and IV PC>TM>IM
Tidemark: no staining; osteocytes and bone matrix: no staining.
Neuraminidase: marked reduction in matrix staining and increase in CM and CY.
β elimination: marked overall increase in matrix staining, decrease in chondrocyte staining.

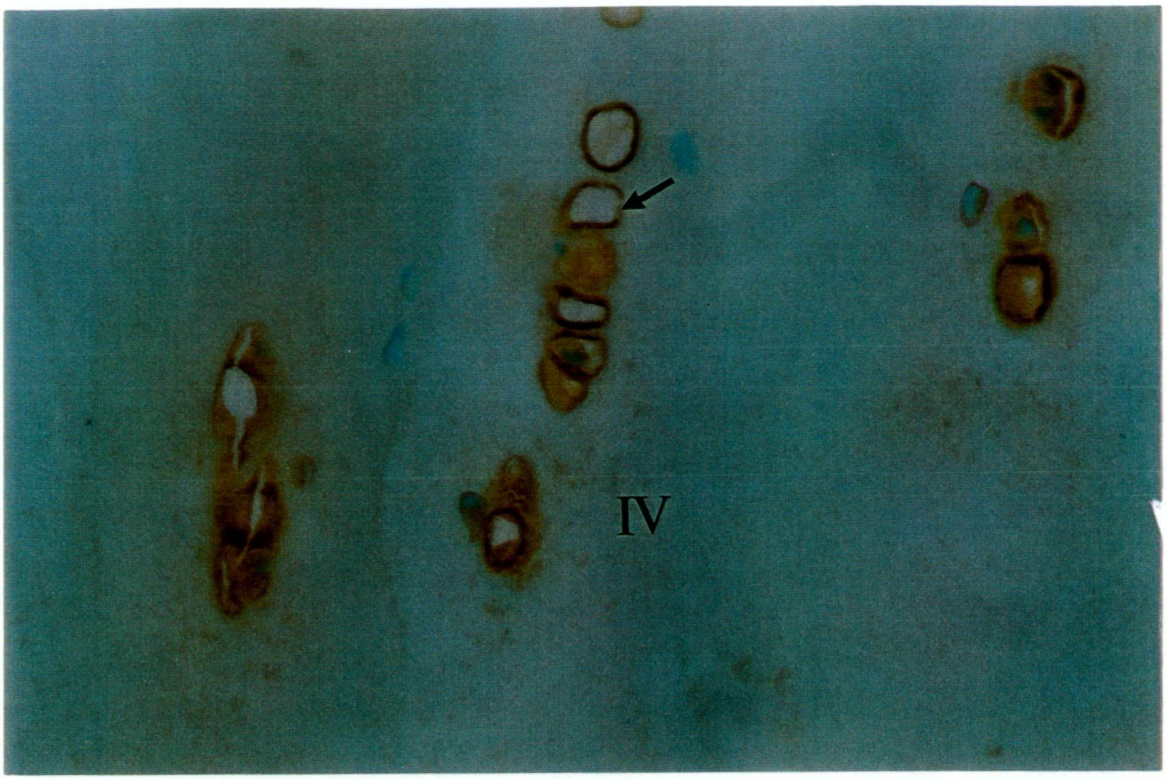


Figure 4-107 SNA Lectin Staining in Normals

Very strong matrix and membrane staining in columns in zone IV. Arrow points to membrane.

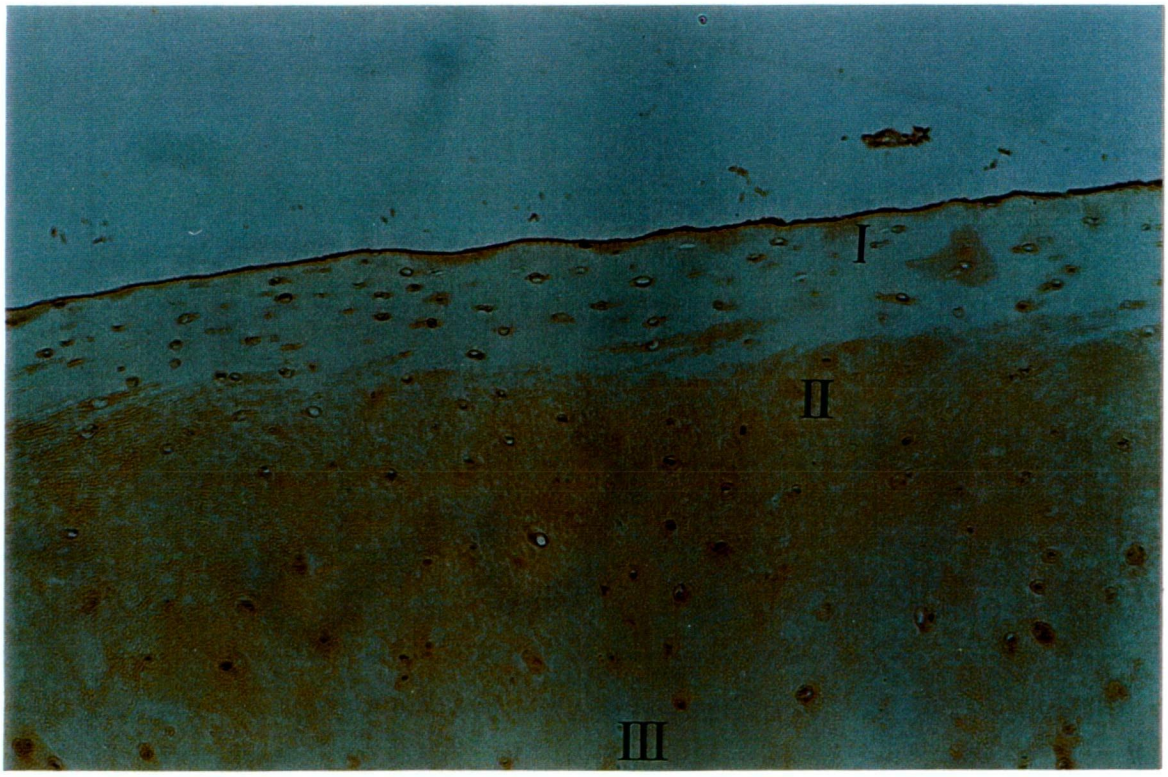


Figure 4-108 SNA Staining in Normals

Strong surface and chondrocyte staining, coarse granular matrix staining.

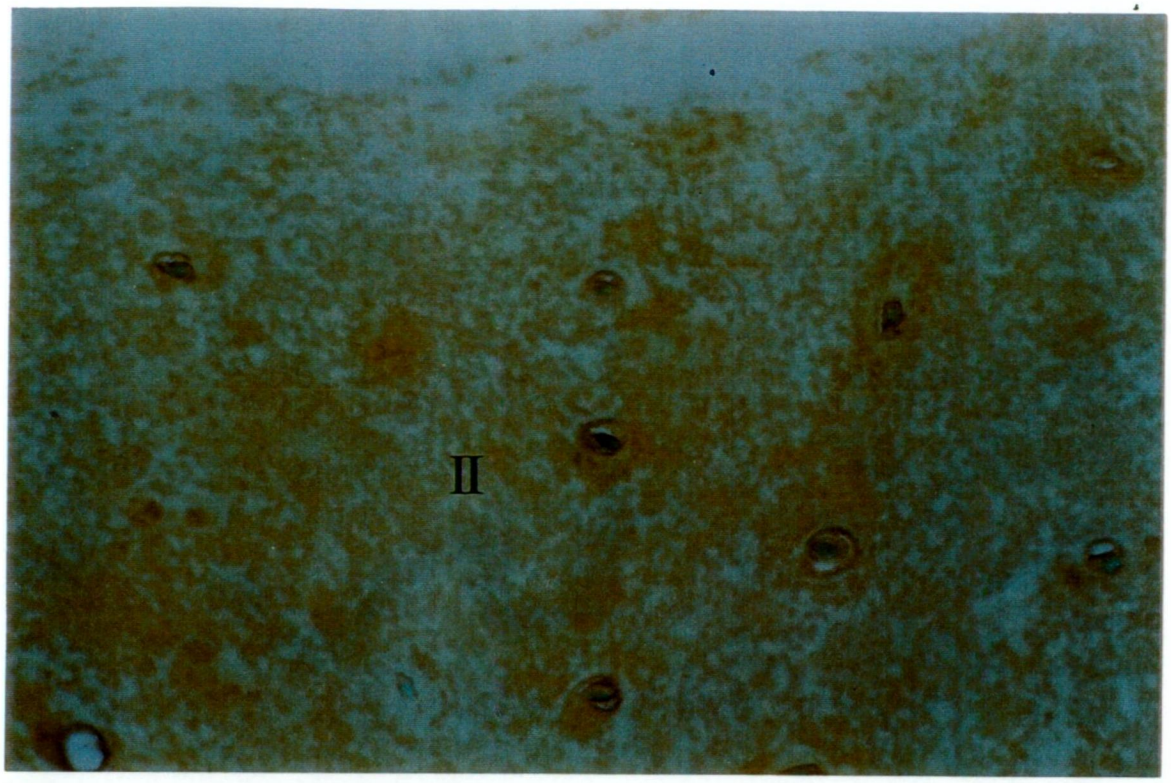


Figure 4-109 SNA Staining in Normals

High power view of zone II to show granular nature of matrix staining.

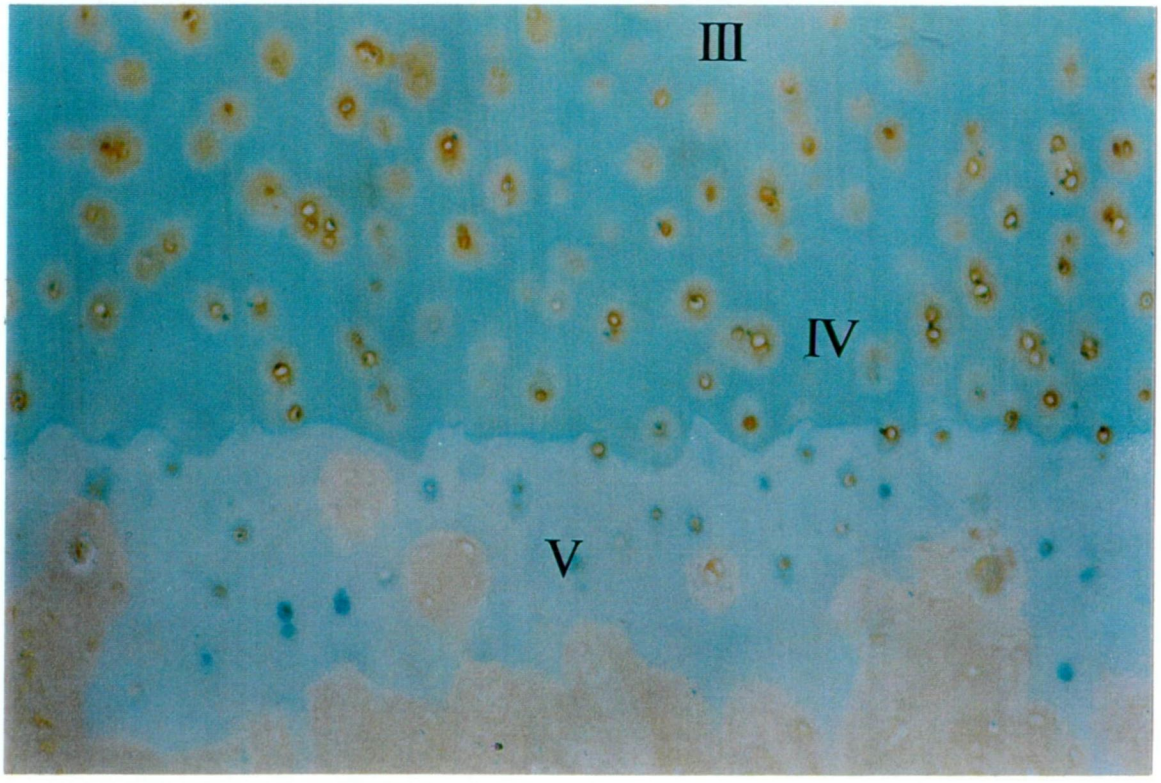


Figure 4-110 SNA Lectin Staining in Normal with Neuraminidase

Very profound decrease in matrix staining with an increase in membrane and cytoplasm staining.

4.7.7.3 LFA

SURFACE: moderate staining was present in the normal and ageing groups. Loss of staining intensity in the early and mild OA groups.

CHONDROCYTES: In zone I the normal, ageing and early OA groups staining of the cytoplasm was absent and that of membrane was virtually absent. In zone II cytoplasm staining was absent in the normal and early OA groups and mild and patchy in the ageing group. In the moderate and severe groups, zone I and II staining was mild and patchy. In zones III and IV in the first two groups there was mild cytoplasm and membrane staining in 20-40% of cells. In the early OA group zones III and IV showed mild cytoplasm staining in up to 60% of cells with absence of membrane staining. In the moderate OA groups cytoplasm and membrane staining was mild to moderate in zones III and IV, with zone IV showing slightly less intense staining in less than 20% of cells. In the severe group, there was moderate cytoplasm and membrane staining in a small percentage of cells in zone III only. Chondrocyte pegs exhibited patchy staining in up to 60% of cells in the ageing, early OA and mild OA groups. Where present, cell membranes of chondrocyte clones showed mild to moderate staining in the majority of cells.

MATRIX: the pattern was the same in all groups in zones I, II and III there was mild matrical staining with none in zone IV (in severe OA the loss extended into zone III). Zone V, peg matrix, intraclonal and periclonal matrix did not stain.

CHONDRO-OSSEOUS JUNCTION: no staining.

SUBCHONDRAL BONE: osteocytes positive, matrix negative.

NEW CARTILAGE: some staining.

ENZYMATIC DEGRADATION:

Neuraminidase: there was a mild increase in chondrocyte staining.

β elimination: overall increase in matrix chondrocyte, osteocyte and matrix staining (matrix I + II > III + IV, PC>TM>IM).

A summary of the key findings is provided in Table 4-24.

Table 4-24 Summary of LFA Lectin Histochemistry Group 7: Sialic acid

OA GRAD	SU	ZONE I			ZONE II			ZONE III			ZONE IV			ZONE V /BONE		
	S	CY	CM	MA	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	CY/CM	PC/TM	IM	TD	MA	OS
0 NORM	2	0	0	1	0/1	1/1	1	1/1	1/1	1	1/1	0/0	0	0	0	1
1 EARLY	2	0	0	1	0/0	1/1	1	1/0	1/1	1	1/0	0/0	0	0	1	1
2 AGED	2	0	0	1	1/1	1/1	1	1/1	1/1	1	1/1	0/0	0	0	1	1
3 MOD	2	1	1	1	1/1	1/1	1	1/1	1/1	1	1/1	0/0	0	0	1	1
4 SEVER	2	1	1	1	1/1	1/1	1	1/1	1/1	1	0/0	0/0	0	0	1	1

Abbreviations: surface (S); chondrocyte cytoplasm (CY), membrane (CM); cartilage regions, pericellular (PC), territorial matrix (TM), interterritorial (IM); tidemark (TD); osteocytes (OS)

COMMENTS

Chondrocytes: overall CY was mild in nearly all groups and zones, apart from zone I in normals, ageing and EOA in zones IV in SOA. CM was seen in a similar distribution.

Matrix: was mild in all groups in zones I – III and absent in zone IV.

Tidemark: did not stain. Osteocytes were positive and bone matrix negative.

Neuraminidase: mild increase in chondrocyte staining, matrix unaltered.

β elimination: overall matrix PC>TM>IM. Overall chondrocytes and osteocytes.

4.8 SUMMARY OF RESULTS

This section provides a summary of the most relevant findings in each of the sections of this thesis.

4.8.1 MACROSCOPIC MORPHOLOGY

Clinical evaluation of patients with unicompartmental and bicompartmental disease appears to be unreliable. Where severe or moderate disease was recorded as unicompartmental clinically there was invariably change in the other compartment. This change macroscopically showed features typical of early OA and this pathological process appears to be macroscopically distinct from ageing. In ageing the cartilage appears yellow and remains relatively firm with mild fibrillation and little response to indentation and a good viscoelastic response. This was in contrast to early OA cartilage where the cartilage remained white but appeared translucent with no or minimal fibrillation and showed a poor viscoelastic recovery to gentle in vivo indentation tests.

4.8.2 MICROSCOPIC MORPHOLOGY

These studies reconfirmed the well documented classical histological descriptions of normal cartilage and severe osteoarthrosis. There were, however, characteristic differences between ageing and early osteoarthrotic cartilage. These changes were seen throughout the cartilage and appeared to demonstrate distinctive pathological processes. In early osteoarthrosis relative to ageing cases there appeared to be much more profound disruption of the chondrocytes in all zones. There was more frequent early clone formation and chondrocyte dropout. The tidemark was more severely disrupted in early OA with multiple tidemarks and frequent vascular invasion. Glycosaminoglycan stains appeared to demonstrate a more profound loss of staining in the superficial zones in the early osteoarthrotic group (a finding consistent with the poor viscoelastic response).

4.8.3 TIDEMARK

The normal tidemark showed very characteristic staining patterns with classical and glycosaminoglycan and collagen stains. With H & E there was a typical basophilic line and with both Toluidine blue and Safranin O there was consistent typical staining whereas with Alcian blue at all pH concentrations there was no staining. These findings are at variance with previous studies as they suggest the presence of some glycosaminoglycans in the tidemark. The collagen stain Picrosirius red demonstrated a distinctive red line adjacent to which was a hazy yellow line suggesting a change in pattern in collagen distribution in this region.

Classical staining with H & E appeared to demonstrate pegs of uncalcified cartilage faithfully followed by the tidemark which dipped into the tidemark and abutted onto marrow spaces. Both physical and computer reconstructed 3D models were able to confirm these microscopic observations. Little attention has been paid to the tidemark region and its role in cartilage homeostasis and nutrition. These studies suggest that this is a region worthy of closer study in relation to disease processes affecting cartilage.

4.8.4 S100 PROTEIN STAINING

The characteristic pattern of staining seen in normals was disrupted in early and severe OA with very variable matrical staining and increased staining around clones. Interestingly the tidemark did not stain.

4.8.5 LECTINS

This next section summarises the key features in relation to each lectin group. The discussion centres on the staining characteristics of lectin groups as a detailed discussion of the significance of these staining patterns in relation to cartilage carbohydrate chemistry is considered in the next chapter.

4.8.5.1 Initial Lectin Trial

The following lectins did not stain Group 3 (BSA II and PWM), Group 5 (SBA) and Group 8 (BSA B4). In Group 1 the staining patterns of GNA and NPA were identical (GNA used in main trial). In Group 2 LCA and ConA showed a very similar pattern

of staining (LCA used in the main trial). In Group 4 and Group 6, AHA and UEA respectively exhibited very little staining and were not included in the main trial.

4.8.5.2 Group 1 Lectins (GNA)

GNA stained matrix only and there were distinctive changes across the groups. Staining was seen in zone I and II only and this decreased across the groups with less staining in the OA groups. Also there was decreased staining across the regions in zone II, PC>TM>IM. No other parameters stained.

4.8.5.3 Group 2 Lectins (LCA, PSA, EPHA, LPHA)

All four lectins stained the matrix with a moderate staining in zone I and in zones II-IV moderate staining falling off to mild across the regions (PC>TM>IM). With LCA, EPHA and LPHA the staining intensity decreased across all zones with increasing disease whereas PSA exhibited no change. The matrix in zone V and pegs stained with PSA and EPHA only.

The cytoplasm and membrane of most chondrocytes stained with all four lectins, the percentage of cells staining decreased both with the severity of disease and depth within the cartilage. Of note was the loss of membrane staining in severe disease with LPHA. With clones again the cytoplasm and membrane stained with all lectins but membrane staining was lost in severe disease with LPHA.

The tidemark did not stain. The vessels, vessel matrix and splits stained with PSA, EPHA and LPHA and were negative with LCA. Both osteocytes and bone matrix stained with LCA, PSA and EPHA in all groups whereas LPHA exhibited staining in the severe group only.

With foci of new cartilage in severe osteoarthrosis LCA, PSA and EPHA stained all parameters but with LPHA there was lack of membrane staining.

4.8.5.4 Group 3 Lectins (DSA, STA, WGA)

All three lectins showed mild to moderate matrix staining with a decrease in staining intensity being seen across the regions PC>TM>IM. All lectins show a decrease in staining with osteoarthrosis. This was most marked with WGA where virtually no staining was seen in zones III and IV. Zone V matrix staining was absent with STA and moderate in normals with DSA and WGA, however, with staining advancing OA with DSA this decreased with WGA increased. The matrix of pegs stained with DSA and STA and was absent with WGA.

Both chondrocyte cytoplasm and membrane stained with all three lectins. The percentage of cells staining decreased in the deeper zones and with more severe disease. The exception was DSA where a greater percentage of cells relative to normals was seen staining in the ageing and early OA groups. Peg chondrocytes showed a variable staining pattern; not all the cells stained and this was variable across the groups. DSA stained all parameters of clones, STA showed a similar pattern but this was more intense with larger clones and WGA only stained in severe disease.

The tidemark stained with both DSA and STA, staining decreasing in the former with disease and increasing in the latter. WGA showed no staining.

All three lectins stained vessels, vessel matrix, splits and new cartilage. With WGA staining intensity increased with the severity of disease. Osteocytes stained with all three lectins and bone matrix with DSA and STA in all groups and with WGA in the OA groups.

With neuraminidase and aryl sulfatase there was increased matrix and chondrocyte staining and this was more pronounced with the former enzyme.
Note: DSA also has affinity with Group 4 lectins and WGA with Group 7, this will be considered in the discussion.

4.8.5.5 Group 4 Lectins (CTA, ECA, MPA)

CTA did not stain the matrix. With ECA there was mild to moderate staining across all zones and regions in normals. There was no differentiation of staining in regions

and relative to the normals staining intensity in the ageing and early OA groups increased in zones I and II and the severe groups there was absent staining in zones III and IV. With MPA in the normals there was moderate staining in all zones, however, as compared to ECA the regions were differentiated (PC>TM>IM). In increasing disease there was a relative increase in staining intensity in the TM and IM regions. Zone V, pegs and clone matrix stained with MPA and ECA only.

A very variable staining pattern was seen with chondrocytes. Both MPA and ECA showed cytoplasm and membrane staining in most cells which became less in advancing disease. With MPA there were less cells staining with advancing disease whereas with ECA this was increased in the moderate OA group and decreased in the severe OA group relative to normals. With CTA chondrocytes did not stain in zone I and II and only the cytoplasm stained in zones III and IV. Peg chondrocytes stained with MPA in the normal group only, moderately with ECA in all groups and with CTA only cytoplasm was stained. With clones staining with MPA was variable, with ECA moderate and again with CTA only cytoplasm stained.

At the chondro-osseous junction CTA and ECA did not stain. MPA showed moderate tidemark staining but did not stain vessels, vessel matrix or splits except in the severe group (ECA showed some tidemark staining in the OA groups).

Osteocytes and bone matrix stained with MPA and ECA but not CTA. New cartilage showed staining of cells and matrix with MPA and ECA but with CTA only cell cytoplasm stained.

Enzyme degradation exhibited significant differences in staining pattern. With neuraminidase there was increased matrix and chondrocyte staining with all lectins. The β elimination procedure led to increased matrix and decreased chondrocyte staining with ECA and MPA. Fucosidase caused increase membrane and cytoplasm staining in ECA and CTA, more marked in the OA groups. With ECA where fucosidase and neuraminidase were combined there was an increase in both matrix and chondrocyte staining. The β galactose procedure with MPA led to decreased

staining of all parameters with the OA groups. Note: MPA also has staining affinity with Group 5 lectins and this will be considered in the discussion.

4.8.5.6 Group 5 Lectins (DBA, VVA B4, WFA, HPA)

All four lectins exhibited matrix staining. With HPA and DBA there was no distinction of regions staining intensity decreasing with HPA and increasing with DBA as disease increased. With VVA B4 there was staining in zone I and II and PC only in zones III and IV and an increase in staining with early OA but a decrease with ageing and severe OA groups. WFA staining zones I to IV with decreasing intensity and increasing disease and there was distinction of regions (PC>TM>IM). Zone V peg and clone matrix were negative with DBA, VVA B4 and HPA and stained mildly with WFA. These subtle differences in staining reflecting the slight variations in affinities that the lectins in this group exhibit

A variable staining pattern was seen with chondrocytes. DBA did not stain the membrane only cytoplasm in a few cells. VVA B4 only stained membranes in severe disease. Both WFA and HPA stained membrane and cytoplasm and there was a slight increase in the intensity and percentage of cells with disease. With clones DBA stained the severe group only, VVA B4 did not stain, WFA was variable and HPA stained cytoplasm only except for the severe group where there was also some membrane staining.

The tidemark stained with HPA but not DBA, VVA B4 or WFA. Vessels, vessel matrix and splits did not stain with DBA and VVA B4, and with WFA and HPA showed moderate staining that increased with groups.

Osteocytes stained with WFA only and bone matrix with WFA and HPA.

Neuraminidase led to increased matrix staining in all groups, with DBA and VVA B4 the percentage of cells staining increased, with WFA it decreased and with HPA it was unaltered. The β elimination procedure led to increased staining of matrix and cells with DBA, VVA B4 and WFA and a decrease with HPA. Note: DBA also has affinity for Group 6 lectins and this will be considered in the discussion.

4.8.5.7 Group 6 Lectin (LTA)

The matrix showed moderate staining in zone I and differentiation of the staining across the regions in zones II – IV (PC>TM>IM) with a decrease in overall stain with increasing disease. Zone V matrix staining was lost in severe OA. Peg matrix stained in all groups whereas clone matrix stained in the severe group only.

Chondrocytes showed no staining in zone I, cytoplasm only in zone II and cytoplasm and membrane in zones III and IV. No cells stained in the severe group. Peg chondrocyte and clone staining was variable.

With fucosidase there was increased matrix staining less marked in the severe groups and a very marked increase in cytoplasm and membrane staining was seen particularly in zones III and IV. β elimination led to increased matrix staining and cytoplasm staining in the latter particularly in zones III and IV.

The tidemark and osteocytes did not stain and matrix showed mild staining only. No parameters at the chondro-osseous junction stained.

4.8.5.8 Group 7 Lectins (MAA, SNA, LFA)

All three lectins showed mild to moderate staining of all zones and regions with a decrease in staining with increasing disease. Pegs, zone V and clones did not stain with MAA and LFA and only mildly with SNA.

In both MAA and SNA cytoplasm and membrane staining was present in all zones and regions in normals. In MAA chondrocytes stained less in the ageing group relative to the OA group. In SNA there appeared to be a slight increase in parameters with increasing disease. In LFA there was a complex pattern of staining; overall there appeared to be more cells staining in zones III and IV in the normal, ageing and early OA groups but only mildly whereas in advancing disease less cells stained with a greater intensity. Clones stained in all groups.

The tidemark did not stain in any of the groups. In MAA and SNA staining of vessels, vessel matrix and splits was present but variable in LFA there was no staining. Osteocytes stained in all groups, matrix staining with MAA and SNA only. There was some staining of all parameters in new cartilage in all groups.

Neuraminidase showed a marked increase in matrix and chondrocyte staining with MAA and SNA but only in chondrocytes with LFA. With β elimination there was an overall increase in matrix staining. With MAA and LFA there was a marked increase in chondrocyte staining particularly with LFA whereas with SNA there was less staining.

4.9 Summary

The results showed characteristic differences in both the macroscopic and microscopic morphological features of ageing and early osteoarthrotic cartilage. A study of the tidemark demonstrated a very characteristic staining pattern with glycosaminoglycan and collagen staining. Three dimensional reconstruction of this region illustrated uncalcified cartilage pegs faithfully followed by the tidemark abutting onto the underlying subchondral bone marrow space.

A detailed study using lectin histochemistry showed that the Group 1-8 lectins all exhibited very characteristic staining patterns in normality and disease. The interpretation of these findings and their relevance to our current understanding of cartilage anatomy and the evolving osteoarthrotic process are discussed in the next chapter.

5 CHAPTER 5: DISCUSSION

5.1 Introduction

The major findings reported in this thesis are:

1. There are characteristic macroscopic and light microscopic differences between ageing and early osteoarthrotic cartilage.
2. The anatomy of the chondro-osseous junction is more complicated than previously documented. The study suggests that interdigitating pegs of uncalcified cartilage faithfully followed by the tidemark, dip through the calcified zone and abut onto the underlying marrow spaces. These pegs are visible on light microscopy and three dimensional reconstruction confirms there is continuity from the uncalcified zone through to bone marrow spaces. Lectin histochemistry with WGA, HPA and MPA demonstrates that cytoplasmic dendritic projections of osteocytes are seen adjacent to both marrow spaces and the uncalcified cartilage pegs. These findings indicate that this region is probably more involved in cartilage nutrition and possibly the evolution of osteoarthrosis than previously postulated.
3. The tidemark has a distinctive pattern of lectin staining with externally and internally located N-acetyl lactosamine, Di-N-acetylchitobiose, β-galactose 1,3/4 N-acetyl galactosamine and α-N-acetyl galactosamine sequences.
4. After S100 protein studies there is increased staining of chondrocytes, clones and pericellular matrix in early osteoarthrosis suggesting that this protein may be involved in the evolution of osteoarthrosis. No staining of the tidemark was seen.
5. Lectin histochemistry of osteochondral sections using a panel of 19 lectins from seven groups showed a very varied pattern of carbohydrate expression.

- In the matrix there were differences in both anatomical zones and regions in the normals, the pattern of expression alters in ageing and osteoarthrotic cases. These features indicate significant changes occur to carbohydrate sequences expressed in the cytoskeleton on proteoglycans and other molecules which currently remain undefined.
- Chondrocyte cytoplasm and membrane has a broad spectrum of staining. In particular chondrocytes appeared metabolically active or in a non-responsive state.

The percentage of cells not expressing lectins is greater in the deeper zones and in osteoarthrosis. These findings indicate that there are significant differences in chondrocyte function in normal, ageing and osteoarthrotic cartilage.

- Osteocytes show a very characteristic staining pattern with a wide range of lectins from groups 1 – 5. The lectins WGA, MPA and HPA are of particular interest demonstrating clearly the intercellular cytoplasmic dendritic processes of osteocytes.

These observations are now discussed in light of current and past research.

5.2 Cartilage Morphology

The present study reaffirmed the classical macroscopic features described in the developing OA process (Hein 1926, Fisher 1929, Collins 1949). There was a significant difference in appearance of cartilage that was "ageing" versus that with "early OA". In the former the cartilage appeared yellow, slightly opaque and exhibited a moderate viscoelastic response to an indentation test (when the surface of normal articular cartilage is indented by gentle pressure it rebounds to its normal shape immediately – a normal viscoelastic response). Whereas "early OA" cartilage was white, opalescent and slightly translucent with a relatively slow viscoelastic response on indentation. This finding was further strengthened by intra-operative indentation tests on early OA cases undertaken while collecting material from arthroplasty procedures (personal observation). This macroscopic observation suggests there may be some anatomical differences between normal, ageing and early OA cartilage. Similar features were found in an experimental canine model of osteoarthrosis after knee joint immobilisation (Jurvelin 1983). Macroscopic observations made on moderate and severe OA differed little from previous studies (Byers 1970, Meachim 1963, 1972, 1984).

Review of the microscopic features of cartilage showed there was a variable pattern of pathology between different regions sampled from the same joint:

1. With bicompartmental, moderate or severe OA affecting the weight-bearing tibial or femoral surfaces, early OA changes were seen in the non-weight bearing posterior femoral components of the joint.

2. With unicompartmental, moderate or severe OA there were frequently early OA changes on the weight- and non-weight bearing surfaces of the contralateral compartment.

From these observations it is evident that the OA process starts in one area of the joint and progress to involve the entire joint suggesting that alterations in joint biochemics and chemical mediators may both lead to altered chondrocyte function.
Osteoarthrosis appears to evolve through a clearly defined early OA phase which can be characterised microscopically as early, moderate and severe OA. The next sections discuss the results of microscopic observation following histochemical staining.

Surface: Microscopically normal cartilage had a smooth surface. In ageing there was some fibrillation and with OA initially there was very mild fibrillation which progressed to deep fissures and eventual cartilage loss. These findings are no different to earlier studies (Freeman 1972, Gardner 1991, Meachim 1973, 1974, 1976, Byers 1970). Some authors suggested that in ageing cartilage fibrillation was non-progressive whereas that of OA were progressive (Byers 1970, 1974, 1976, 1977, Mitrovic 1987). In the current study it was not possible to confirm or refute these observations.

Matrix: In normal cartilage there was a uniform pattern of matrix staining slightly less intense in the most superficial and deep parts of the cartilage. With ageing and early OA these areas of less intense staining receded towards the middle regions of the cartilage. In those cases classified as early OA with mild/nil surface change the degree of pallor was greater than in the OA group. With moderate and severe OA there was a very marked overall loss of staining with focal increases in the vicinity of clones. With alcian blue at varying pH's there was loss of chondroitan sulphate from zones II and III and an increase in keratan sulphate in the deeper zones. These observations do not differ significantly from other works, however, little emphasis appears to have been placed in the marked difference in staining between ageing and early OA cartilage (Mankin 1971 A & B, Inerot 1978, Vasan 1980, Elliott 1979, Korver 1990).

Matrix Collagen: There was fairly uniform staining with PSR with a slight increase in intensity at the surface and pericellular regions. In severe OA there was marked

disruption of the cartilage matrix staining with areas of high intensity around some clones. These findings are consistent with normal cartilage architecture and the known matrix changes that occur in OA (Broom 1982). Whether the changes in appearance reflect altered collagen ultrastructure and/or altered collagen types need more extensive study with appropriate markers for the different collagen types. Certainly altered collagen expression is reported in developing OA with increased amounts of type II and X collagens (Adam 1983, Lippiello 1977).

Chondrocytes and Clones: After semiquantitative assessment there appeared to be differences in chondrocyte architecture in early OA and ageing cartilage. In early OA cartilage with an intact surface the chondrocyte architecture was disrupted, upper zones showed dropout of cells and prominent early clone formation, and in the deeper zones there was disorganisation of the normal cell columns. The changes in ageing cartilage even with some surface disruption were not as marked. In severe OA huge multicellular clones were seen in areas of fibrillation with up to 30 – 40 chondrocytes, adjacent cartilage was acellular with virtually no staining for glycosaminoglycan. In some areas in early and moderate OA a few chondrocytes became "hypertrophic" like the cells of the growth plate (Brighton 1978, Ozawa 1984). Chondrocytes in zone V and pegs did not form clones or become hypertrophic.

These findings are in general agreement with other studies which show marked early changes in OA in proteoglycans (Dunham 1990, Vasan 1980) collagens (Broom 1982) and chondrocytes (Rothwell 1973). The synthetic (Treadwell 1985) and phenotypic modulation (Archer 1990) of sub-populations of chondrocytes is poorly understood in normality and disease. Chondrocytes are constantly remodelling the matrix in which they are suspended in response to physical and chemical changes within the environment. In experimental studies heat (Madreperla 1983), load and pressure affect the synthesis of matrix protein and the degradation rate of the matrix (Lipiello 1977, 1985). In understanding the physiology of the matrix the concept of the chondron, that is the chondrocyte and its specialised pericellular microenvironment, has proved useful (Poole 1987, 1991). In both ageing and osteoarthritis there are very marked alterations in chondrocyte morphology and cell density (Mitrovic 1983) and chondrocyte replication termed clone formation

(Rothwell 1973). All these features indicate the importance of understanding the normal biosynthetic functions of chondrocytes.

Chondro-Osseous Junction: There was very marked disruption of the chondro-osseous region in both early OA and ageing, however, the changes were more prominent in the former. Early duplication and triplication of the tidemark was seen along with prominent vascular invasion. In moderate and severe osteoarthrosis there were increasing numbers of tidemarks and the degree of disruption was such that distinguishing between calcified and uncalcified cartilage and bone was difficult to assess. These observations were similar to those made by other authors (Lane 1980, Teshima 1977, Oegama 1992).

Sub-chondral Bone Plate: In this study as specific bone stains were not used, assessment of bone cells (osteocyte, osteoblasts, osteoclasts) proved difficult. Areas of new cartilage formation were noted within the immediate subchondral area described by some authors as metaplastic cartilage (Schunke 1988). Marrow spaces became fibrotic and in a few cases there were areas exhibiting features of fat necrosis. Occasional foci of woven bone were noted on polarisation and endochondral ossification was not a prominent feature. Only a few cases with very severe OA were collected in this study and although there were clearly marked changes in this sub-chondral region the focus of the study was kept to articular cartilage.

Summary: Cartilage pathology may occur because matrix changes lead to perturbations in chondrocyte metabolism or because either intrinsic or extrinsic factor acting on the chondrocyte alter their metabolic functions. Both these factors may of course be acting in concert. In ageing there may be "normal" subtle changes in the cartilage cytoskeleton which are an inevitable consequence of senescence. Minor disruptions in collagen cross-linking could lead to alteration in matrix hydration and consequently chondrocyte metabolism. In early OA for example marked changes were seen in the non-weight bearing cartilage from the posterior condyle in joints with established OA in other anatomical area. This cartilage shows all the hallmarks of early OA and it may be that there were primary changes to the chondrocytes. Chemical mediators present in the synovial fluid may diffuse into the matrix and lead to secondary alterations chondrocyte function. This would trigger a snowball effect

leading to matrix changes and consequently secondary affects on the chondrocytes. There were characteristic cellular differences between ageing and early OA cartilage and these changes may reflect different pathophysiological processes. One the consequence of the natural ageing process and the other due to a disease process i.e. osteoarthrosis.

5.3 Tidemark

In adult articular cartilage, the interface between the calcified and uncalcified cartilage is a thin clearly demarcated basophilic line on H & E staining. Because of the relative paucity of research clearly defining the structure of the tidemark in normality and disease this study undertook to review the lectin and histochemical properties of the tidemark in decalcified human knee joint cartilage. In addition, the microanatomy of the tidemark in normal cartilage was defined by both physical and computer generated reconstruction techniques. A revised model of the anatomy of the tidemark region is proposed in light of these observations.

5.3.1 Histochemistry Observations and Interpretation

Histochemistry: The current study confirms the classic description of the tidemark in normal joints as being a basophilic line on H & E staining (Fawns 1953) separating uncalcified and calcified cartilage (Thomson 1882, Meachim 1963). With toluidine blue the tidemark was seen as a crisp deep navy blue line, with safranin O as a bright orange-red line and with alcian blue at varying pH's there was no staining. Previous studies have not undertaken PSR staining which demonstrated the tidemark as a bright red line. Other studies with collagen stains showed positivity with phloxine and tartrazine (Lendram's stain) in both calcified and uncalcified tissue (Dmitrovsky 1978, Fawns 1953) and negativity with Masson's trichrome (Green 1970).

These results suggest that the tidemark is rich in proteoglycans, however, the lack of staining with alcian blue at variable pH suggests there are no keratan sulphate or chondroitin sulfate side chains. Strong collagen staining with PSR confirms the tidemark is distinct from the adjacent calcified and uncalcified regions in regard to its collagen ultrastructure. Scanning electron microscope (SEM) studies suggested

vertically orientated collagen fibres run from the uncalcified cartilage and were "anchored" in the calcified cartilage (Sokoloff 1979, Inoue 1981). The tidemark region was distinguishable as the interface between these two zones (De Bont 1986). Collagen fibrils were noted to be mixed in irregularly amongst vertical fibrils at the tidemark (Inoue 1981) and intervening spaces were filled with non-fibrillar material extractable with trypsin or hyaluronidase (Hough 1975, De Bont 1986). These ultrastructural studies suggest the tidemark to be a distinctive collagen and proteoglycan rich zone findings confirmed by the histochemical staining features seen in this study.

Mineralisation: The tidemark has been considered functionally to represent a mineralisation front separating calcified and non-calcified zones (Boskey 1980) and has been demonstrated to contain matrix vessels (Teshima 1977, Bullough 1983, Rees 1988). These vesicles have been shown to act as hydroxyapatite nucleators and the tidemark appears to be intimately involved in the matrix-matrix interactions that must be occurring in this zone of transition from uncalcified to calcified cartilage (Boskey 1976, 1981, 1987). The use of tetracycline which is incorporated at sites of calcification deposition have confirmed this region has a high level of metabolic activity (Lemperg 1971, Revell 1990). It is postulated that the cytoskeleton formed by the collagen fibres and entrapped glycoproteins at the tidemark have a significant role in correct orientation of the extracellular matrix vesicles.

Uncalcified cartilage pegs: Previous research has focused on the ultrastructure of the tidemark (Inoue 1981), its tinctorial properties (Green 1970, Dmitrovsky 1978) and its role as the mineralisation front (Boskey 1980, Teshima 1977). Very few studies have focused on its overall anatomy and the significance that this might have on its normal physiological role. Some authors have considered it as a membrane that runs across the joint and merges with the periosteum (Havelka 1984 & 1986) and others as a zone of least resistance (Spinelli 1976). In the present study re-interpretation of light microscope features suggest that the tidemark is a much more complex region than has been commonly accepted. Pegs of uncalcified cartilage interdigitated with the calcified cartilage and these were faithfully followed by the tidemark and in places abut onto marrow spaces (Figures 4-44 to 4-66). That uncalcified cartilage was seen

in contact with marrow spaces is important in relation to cartilage nutrition as our knowledge on this subject is not complete.

Nutrition: Besides the connections with marrow spaces via the uncalcified pegs reported in this study there are other vascular links between cartilage and bone that may have a metabolic role. Nutrition of articular cartilage has been a long debated subject and studies have been undertaken on both humans (Greenwald 1969) and rabbit cartilage (Hodge 1969, Honner 1971, Redler 1975, Havelka 1986). These authors reported vascular channels that run up through the calcified cartilage into the uncalcified cartilage (Clarke 1990). The existence of such vascular channels suggests that cartilage receives nutrition via both synovial fluid and the underlying subchondral bone. Other authors consider that the tidemark acts as a barrier to nutrition (Collins 1949, Ekholm 1951) and studies using radiolabelled hydrogen suggested that the transfer of solutes across this region was limited (Ogata 1979).

A number of studies have alluded to variations in thickness of the uncalcified zone and to contacts with vascularised underlying "woven" bone (Woods 1970, Pedley 1979, Meachim 1984, Muller-Gerbl 1987, Gilmore 1987). Three types of contact between uncalcified cartilage and bone have been described in human femoral head cartilage. These are, Type A discontinuity in the tidemark with contact of soft tissue and uncalcified cartilage, Type B contact with soft tissue encased in woven bone and Type C contact with small vascular core. (Meaham 1984, Woods 1970). The current studies confirm the presence of both A, B and C type contacts and vascular channels in normal cartilage. However, these are a variable feature occurring infrequently as opposed to the presence of interdigitating uncalcified cartilage pegs which are a consistent feature. These cartilage pegs appear present in photographs in a number of papers (Woods 1970, Redler 1975, Gilmore 1987), however, more emphasis has been placed on the contacts and vascular channels.

Mechanics: Besides its proposed role in mineralisation and nutrition, the tidemark region may play a crucial role in the transmission of forces from articular cartilage to the subchondral bone (Broom 1982A). It has been argued that forces are transmitted along vertically orientated collagen fibres (Sokoloff 1973, Redler 1975, Haynes 1981) and that the tidemark might have a role in this process. Micromechanical studies

undertaken by Broom (1982A) suggest that if anything the tidemark might act to constrain the transfer of mechanical forces. He argues that the response of the tidemark is a function of "(a) the local form and orientation of the tidemark, (b) the organisation of the collagen fibres, (c) the position of this region with respect to the compressive anvil". The presence of splits at the tidemark might argue in favour of Broom's (1982A) studies and suggest that it is a line of weakness. The present study demonstrated that there were horizontal splits at the tidemark, particularly in early OA and ageing cartilage, and confirm observations made in other studies (Meachim 1978). These splits were not seen in moderate or severe OA when this area was totally disorganised which suggests the mechanics of the area has altered.

Ageing and OA: The tidemark appears to be a metabolically active region in both normal, ageing and osteoarthrotic cartilage (Revell 1990). It shows significant pathological changes with ageing when tidemark reduplication occurs even in the absence of fibrillation (Green 1970, Meachim 1984). With developing osteoarthrosis there was increasing disruption of the tidemark with the formation of multiple tidemarks (Lane 1980). Although early in the osteoarthrotic process the calcified cartilage zone remains fairly consistent in thickness (Meachim 1984) with increasing severity of disease there was increasing disruption. Multiple tidemarks co-exist and the clearly defined boundaries across the uncalcified-calcified-cartilage-subchondral bone interfaces are disrupted. There was greatly increased vascular "invasion" into the area and distinction into functional areas was difficult. Many of the vascular plugs were characterised by multinucleated giant cells, termed chondroclasts, which were present at the tip of the plugs much like osteoclasts in the "cutting cones" of bone remodelling. This spectrum of changes was seen in the current study and it appears that disruption of this region is more marked in early osteoarthrotic cartilage relative to ageing cartilage. In the former there is more frequent replication of the tidemark and more prominent vascular invasion.

5.3.2 Microanatomy

Because of the 2D observations made on the presence of cartilage pegs dipping into calcified cartilage, a 3D reconstruction of the region was undertaken. This was initially carried out using a simple physical model in which a series of sequential

photographs were taken of normal tidemark region "cut out" and overlaid (Figure 4-47). Straight forward as this technique was it elegantly demonstrated the continuity of pegs through the calcified zone. No research exists using 3D computer reconstruction technique in relation to the tidemark. In collaboration with Hubbold and Hancock (1997, 1999) a 3D reconstruction technique developed for the assessment of "hot" and "cold" spots in radiation dose fields in tumour management was applied to the tidemark region. In this system 50 serial black and white images fed from a microscope mounted computer controlled digital camera allowed reconstruction of the region. Grey scales were converted to colour images and using appropriate computer algorithms this data was rendered into 3D images.

The reconstructions clearly demonstrate a peg of uncalcified cartilage dipping through the calcified cartilage and enveloping a region adjacent to a marrow space (Figures 4-48 to 4-51). Resolution was not sufficient to demonstrate the tidemark as a separate entity, however, histochemical studies indicates that the tidemark always follows these pegs. These findings suggest that the physiological functions of this region are more complex than previously documented. It is postulated that the connections with the marrow space indicates there may be a role in cartilage nutrition. In addition, as this area is profoundly affected in osteoarthrosis it is postulated that humoral mediators may impart a role via these communications on matrix components.

5.3.3 Lectin Histochemistry

In the current study the tidemark stained with the following lectins: DSA, STA, MPA, ECA, HPA. The interpretation of these staining patterns are discussed and consideration is given to the non-staining lectins.

Group 1,2,6,7 lectins: In these groups there was no staining with GNA (Shibuya 1988); LCA, PSA, EPHA, LPHA (Kornfield 1975 & 1981, Debray 1981 & 1983, Cumming 1982, Yamashita 1983); LTA (Petryniak 1986) and MAA, SNA, LFA (Wang 1988, Shibuya 1987, Miller 1982). This suggests the tidemark does not exhibit high mannose, N-linked, fucosyl or sialyl sequences.

Group 3 lectins (DSA, STA, WGA): The presence of staining with DSA and STA suggested the presence of N-acetyl lactosamine and Di-N-Acetylchitobiose sequences. In addition, DSA has some affinity with Group 4 lectins particularly highly branched saccharides with β galactose (Debray 1981, Yamashita 1987). There was no staining

with WGA therefore there were no internal chitobiose, repeating N-acetyl lactosamine or terminal sialyl residue sequences.

Group 4 lectins (CTA, ECA, MPA): There was prominent staining with MPA suggesting the presence of β-galactose 1,3 N-acetyl galactosamine α 1- and α-N-acetyl galactosamine sequences, the latter reflecting homology with Group 5 lectins. No staining was seen with CTA reflecting subtle differences in saccharide subsets, as both CTA and MPA bind N-acetyl lactosamine. With β-galactose staining in normals was unaltered and in OA it was decreased indicating removal or less synthesis of this sugar or increased degradation with exoglycosidases due to alterations in the matrix. The technique of β elimination increased staining indicating no binding to alkali-labile oligosaccharides and/or that binding was to alkali stable N-glycan structures, to odd non-cleaved O-glycans or to undetermined O-glycans that are inhibiting access. With neuraminidase there was increased staining therefore N-sialyl residues may be concealing binding sites (Sarkar 1981, Young 1989), either directly due to covalent bonding or indirectly due to physical hindrance. ECA showed tidemark staining after fucosidase digestion, therefore, fucosyl residues may be concealing underlying β-galactose sequences, either on outer or core structures. As opposed to the β 1,3 linkage ECA has a preferred affinity for the β 1,4 linkage as in the disaccharide Gal β 1,4 GlcNAc β 1- (Iglesias 1982, DeBoeck 1984).

Group 5 lectins (HPA, WFA, VVA B4, DBA): The tidemark stained with HPA with no alterations with either neuraminidase or β elimination, therefore terminal N-acetyl galactosamine sequences are being expressed (Sutton 1992, Torres 1988). Lack of staining with the other lectins may be accounted for either by the location of GalNAc α 1- (internal or external) or by adjacent substitutions.

Summary: There was no expression of mannosyl, complex N-linked, fucosyl or sialyl sequences, whereas there was expression of externally and internally located N-acetyl lactosamine, Di-N-acetylchitobiose, β-galactose 1,3/4 N-acetyl galactosamine and α-N-acetyl galactosamine sequences. Enzymatic degradations using β-galactose, β-elimination, neuraminidase and fucosidase suggested an altered expression of β-galactose residues in OA (decreased synthesis or increased breakdown), an absence of

O-linked oligosaccharides, binding to odd O-glycans or alkali stable structures and that sialyl and/or fucosyl residues are concealing outer or core associated β-galactosyl sequences.

5.3.4 New Model of Chondro-Osseous Region

The current studies focused on a review of the microanatomy of the tidemark based on light microscopic studies using a selection of lectin and histochemical techniques. In addition a 3D reconstruction of the area was undertaken. From these studies it is postulated that the chondro-osseous region is more complex than previously considered. The tidemark itself is a clearly defined boundary delineating uncalcified and calcified cartilage. It is rich in collagens and proteoglycans and expresses a pattern of lectin staining unique to other anatomical areas in the joint. It is not a straight line across the joint but a complex 3-dimensional structure that follows the uncalcified cartilage pegs that dip into the calcified cartilage and abut onto underlying marrow spaces. Blood vessels do extend across this region and alterations in the relative thickness of the zone V cartilage allow for direct contact between bone and uncalcified cartilage (the A,B and C contacts described in Woods 1970). These newly defined uncalcified pegs and other vascular connections would strongly support an argument for a role of the chondro-osseous region in nutrition. Based on the current and previous studies it is therefore proposed this region is involved in joint nutrition, calcification mechanisms and the transfer of forces from hyaline cartilage to bone. The significant pathological changes that occur early in the osteoarthrotic process appear to reaffirm this region's physiological importance.

5.4 S100 Protein Staining

S-100 protein is a group of calcium binding proteins in the same category as calmodulin, troponin C, parvalbumen and intestinal calcium binding protein (Kamegai 1990). In the current study sections of decalcified normal, early OA and severe OA cartilage were examined.

In the normal group there was strong staining of cytoplasm and membrane in all zones and of the pericellular and territorial matrix in zones I and II. These findings concur with the staining patterns noted by other authors (Stefansson 1982, Mohr 1985, Nakamuri 1988). No staining of osteocytes, calcified cartilage or the tidemark was

seen. In the study undertaken by Mohr (1985) there was tidemark staining but no staining of zone V chondrocytes. In both early and severe OA there was an increase in staining of chondrocytes, clones and the immediately adjacent pericellular matrix. Matrical staining was otherwise patchy and variable. Both Mohr (1985) and Chen (1990) observed similar features. Increased staining has been seen in areas of chondroid metaplasia (Mohr 1985) and in osteophytes (Chen 1990) but no extensive review of the chondro-osseous region has been undertaken.

Interpretation of these findings is difficult as the exact function of S-100 protein in chondrocytes is undefined. A number of studies on normal cartilage tissue in chondroidal lesions and in osteoarthrosis show S-100 protein expression (Stefansson 1982, Cocchia 1983, Nakamura 1983, Mohr 1985, Weiss 1986, Itoi 1988, Okajima 1988, Kuzuhara 1989, Chen 1990). The protein is also detected in a wide variety of other tissue types particularly from the nervous system (Vinores 1984). Its role in both chondrocytes and other tissue lines however remains open to some conjecture. This protein is clearly expressed by chondrocytes and an increase in staining intensity in OA may be interpreted as increased cellular activity. There is also protein in adjacent matrix most intense in the pericellular region suggesting the protein is excreted from the cell. In the current study tidemark staining was absent, however, vessels and vessel matrix stained extensively across the tidemark. Other studies have reported tidemark staining. Chen (1990) believes this is artefactual while Mohr (1988) considers it to confirm a role in calcification. The lack of staining in the current study may reflect differences in calcification techniques/times or in the immuno-histochemical staining procedures. S-100 protein clearly has a role in defining chondrocyte pathology in normal and "diseased" cartilage, however, its biochemical roles require further defining.

5.5 Lectins

There was a great variation in lectin staining both within cartilage zones and regions and in normal, ageing and osteoarthrotic cartilage. The structure of the cartilage matrix is very complex involving many undefined matrix-matrix and matrix-cell interactions. Lectins provide a usual biochemical tool for defining cartilage carbohydrate ultrastructure in normal cartilage and unravelling subtle changes that

occur in ageing and developing osteoarthrosis. The next sections considers the staining patterns seen with the various lectin groups and discusses the interpretation of these observations in light of the current study and previous research.

5.5.1 Surface

All lectin groups showed moderate to strong surface staining that was unaffected or slightly diminished by ageing or osteoarthrosis. Either this reflects that multiple carbohydrate residues are being expressed or this is an artefactual edge effect. No previous studies have considered this feature.

5.5.2 Matrix – Zone I-V, Pegs and Clones

Introduction: The staining pattern in the matrix was variable across the groups. Overall zones I and II showed the most intensity with a decrease in expression of residues further away from the chondrocytes (PC>TM>IM).

Group 1 lectin (GNA): There was moderate staining in zones I and II with GNA with decreased expression across the regions (PC>TM>IM). Therefore mannosyl residues are being expressed in the matrix but not by the chondrocyte. Staining intensity was diminished in osteoarthrosis indicating this may either be a reflection of increased hydration or an decrease in the production of mannose containing oligosaccharides. There was no staining of zone V, pegs or clone matrix. This lectin has not been used in this field of research previously.

Group 2 lectins (LCA, PSA, EPHA, LPHA): Lectins showed moderate staining of zones I to IV. LCA, EPHA and LPA show decreased staining in ageing and OA, whereas PSA was unaltered. This implies the matrix has a broad expression of complex N-linked glycans including, non-bisected bi/tri/tetra antennary N-glycans. Decreased staining with OA may reflect increased matrix hydration. PSA recognises less arborised glycans and these may be unaltered in the OA process or possibly even increased to compensate for a reduced matrix concentration due to hydration. The findings in the current study are similar to observations made by Picton (1988), although this author found that LPHA showed increasing IM staining over TM

staining possibly due to differences in fixation. Both PSA and LCA bind to very similar biantennary structures (Debray 1981, Kornfield 1981) with the highest affinity where α 1,6 fucosyl residues are attached to asparagine linked N-acetyl lactosamine. The difference in expression in ageing shows that LCA prefers structures with longer antennae than PSA (Yamamoto 1981, 1982). These lectins also bind to triantennary sequences especially where one of the outer α -mannose residues is substituted at C-2 or C-6 and/or there is a core fucose (Kornfield 1981). Therefore LCA and PSA are detecting very similar subsets of N-linked oligosaccharides, the latter with shorter antennae.

EPHA binds with greatest affinity to bi/tri antennary oligosaccharides with a bisecting N-acetyl glucosamine residue. The antenna arising from C-6 to the core β mannose is important in binding and sialyl residue free (Yamamoto 1981). LPHA has the greatest affinity for non-bisected tri/tetra antennary sequences with outer galactose residues and an α -mannose substituted at C-2 and C-6 (Cummings 1982, Hammastrom 1982). EPHA and LPHA have distinct binding patterns as a result of these different subsets.

The clonal matrix showed the same pattern of staining suggesting a similar distribution of complex N-linked glycans. Neither zone V or pegs stain therefore they do not express complex N-linked glycans.

Group 3 lectins (DSA, STA, WGA): Showed mild to moderate staining of all zones which was decreased in the TM and IM, and in ageing and OA. In OA these changes were most pronounced with WGA where no staining was present in zones III and IV. Zone V matrix staining was moderate in normals with DSA and WGA, decreasing in the former and increasing in the latter in OA. STA did not stain Zone V. Clone matrix stained with all lectins, being most marked in OA with WGA. Pegs stained with DSA and STA but not WGA. These findings indicate expression of N-acetyl lactosamine, di-N-acetylchitobiose, β galactose (DSA group 4 affinity) and sialyl sequences (WGA group 7 affinity). Like WGA, STA has extended binding sites and has hydrophilic areas which align with L-fucose and may interact with terminal N-acetyl glucosamine and sialyl residues at high concentrations allowing it to bind to sugars in keratan

sulphate. WGA binds to terminal sialyl residues (Debray 1981, Bhavindrum, Gallagher 1985, Allen 1973, Goldstein 1981) and also exhibits an affinity for residues on the core of bisected N-linked oligosaccharides (Yamamoto 1981).

Following digestion with neuraminidase and aryl sulfatase, WGA staining was seen in Zones III/IV therefore cleavage of sialyl and sulfate residues is revealing repeating N-acetyl lactosamine core sequences. The subtle differences in staining in this group may be accounted for by the degree of branching of N-acetyl lactosamine and oligosaccharides on keratan sulfate. The pattern of staining with WGA is similar to that seen by Picton (1988), DSA and STA have not been previously used in similar studies.

Group 4 lectins (CTA, ECA, MPA (also Group 5) *DSA*): ECA was expressed mildly to moderately in all zones (less so in III and IV) and regions were not differentiated. In ageing and early OA staining increased and in mild and severe OA it decreased. ECA binds peripherally to subset sequences of N-acetyl lactosamine and also to the β 1,3 linkage analogue of the saccharide (De Boeck 1984, Iglesias 1982, Kaladas 1982, Ehrlich-Rogozinski 1987). Increased staining particularly in the OA groups was seen with ECA after both neurominidase, fucose and both combined. This reflects that removal of fucosyl and sialyl residues both from terminal and core structure are revealing β–galactose sequences probably in N-acetyl lactosamine.

CTA was expressed only after fucosidase pre-treatment. CTA may be binding to core sequences of N-acetyl lactosamine revealed by removal of fucosyl residues (Bhattcharyya 1989).

MPA was expressed moderately in all zones and regions were differentiated (PC>TM>IM). Staining in the TM and IM increased slightly in early OA and ageing but in moderate and severe OA decreased in all zones and regions. This decreased staining in OA again probably reflecting increased hydration. Both MPA and DSA have a similar staining pattern and have a much greater affinity for the 1,3 linkage (Sarkar 1981, Young 1989, Maharta 1999, Bausch 1977). MPA binds at two sites, Gal β 1,3 GalNAc α 1> GalNAc α 1. Therefore MPA also has affinity for the Group

5 lectins which bind N-acetyl galactosamine and consequently the 1,3 linked disaccharide has a perfect match for both sites. The 1,3 linked disaccharide is presented as terminal stubbs and increased binding in TM and IM in OA may reflect increased synthesis of 1,3 over 1,4 linkages. The binding site Gal β 1,3 GalNAc is a recognition site for the Thomson-Fredrickson (T antigen) and this is often masked by terminal sialic acid residues. The aforementioned biochemical feature would account for increased binding after neuraminidase which cleaves sialyl residues. With β elimination there was increased staining suggesting binding to revealed N-linked oligosaccharides or obscure alkali-stable O-linked oligosaccharides. The pattern in OA after β galactose was decreased suggesting a greater accessibility to these groups after the cartilage is disrupted by OA. The matrix of zone V, clones and pegs stained with MPA and ECA but not CTA, suggesting that these areas of matrix contained β galactose.

The pattern of staining with MPA was similar to that seen by Picton (1988). Neither ECA or CTA have been used in osteoarticular studies previously.

Group 5 lectins (HPA, WFA, VVAB4, DBA): None of this group have been used in previous cartilage studies. Both HPA and DBA showed mild to moderate uniform staining of all zones and regions, with DBA this increased in disease and with HPA decreased. WFA showed patterns seen previously with greater staining in zones I and II and a decrease across the regions (PC>TM>IM). The staining pattern decreased in ageing, early and severe OA and increased in mild OA. Both HPA and DBA stain terminal GalNAc α 1- residues. (Etzlar 1970, Torres 1988, Hammastrom 1977, Sutton 1992). In addition, DBA has greatly enhanced binding when there is α 1-2 fucosyl substitution of the sub-terminal galactosyl residue in α 1,3 linkage to the terminal GalNAc as in GalNAc α 1,3 (Lfuc α 1,2) Gal β 1, 3/4 GlcNAc β 1- (blood group A leucoantigen). Its affinity with Group 6 lectins is explained by the bifurcated binding site which has regions for both GalNAc and fucosyl residues. This difference from HPA may account for the differences in staining affinities seen with developing OA. VVAB4 has some similarity with DBA but is seeing large saccharides with more sugar (Sueyoshi 1988), in particular, it recognises structures which are internally O-glycosidically linked.

These lectins all have affinities for the site of GalNAc accounting for the variations in staining. WFA (Sugii 1980) prefers to bind to 1,6 linked over 1,3 linked galactose as in GalNAC α 1,6 Gal β 1- > GalNAc α 1,3 Gal β 1- and therefore the 1,6 linked form may be expressed in the upper zones and pericellular region. Neuraminidase led to increased staining with DBA and VVA B4 therefore removal of sialyl residues reveal β-galactose binding sites. With WFA staining was decreased implying that there were sialyl residues in close proximity to the binding site and the chemistry of the region is altered. HPA was unaffected and therefore has binding sites not affected by sialyl residues. The technique of β elimination led to increased staining of matrix with DBA, VVAB4 and WFA indicating an affinity for alkali stable structure (both N-linked and unusual O-linked oligosaccharides). There was decreased staining with HPA, therefore there may be binding sites on O-linked oligosaccharides. The matrix of zone V, clones and pegs stained with WFA only indicating there were more 1,6 galactose linkages in these areas.

Group 6 lectins (LTA, *DBA* [Group 5]): There was moderate staining in all zones with decreased staining across regions (PC>TM>IM) and the intensity was less with developing disease. The main binding site for this lectin is the fucose residue in α 1,6 linkage to β GlcNAc in N-glycosidic linkage to asparagine but it also binds to fucose residues in α 1,3 linkage to β GlcNAc outer terminals (Petryniak 1986). With fucosidase the increased staining is accounted for by removal of peripheral fucosyl residues allowing exposure of internal core residue or N-glycan. LTA has not been used in previous trials but UEA which stains fucosyl residues was shown to exhibit a similar pattern of staining in other studies (Hoed-Schmidt 1989 A/B). The matrix of zones V, clones and pegs stained with LTA suggesting that there are fucosyl residues (peripheral and core) in these areas.

Group 7 lectins (MAA, SNA, LFA): There was moderate staining of all zones and regions and this was decreased in OA. Therefore the matrix is expressing sialyl residues α 2,3 (MAA) and α 2,6 (SNA) linked to β galactose and N-glycosyl neuraminic acids (LFA) (Wang 1988, Shibuya 1987, Miller 1982). With neuraminidase there was increase in matrix staining with MAA and SNA therefore

they may be binding to exposed core sialyl residues after removal of outer chains. As LFA stains the glycolyl derived acid this was unaltered as they are not cleaved by neuraminidase. After β elimination increased staining indicates binding to exposed N-linked oligosaccharides or non-cleaved O-linked oligosaccharides. The matrix of zone V, clones and pegs stained with SNA thus they may contain α 2,6 linked sialyl residues. Previous studies have not used this group of lectins.

Summary: These results indicate that the carbohydrate chemistry of the extracellular matrix is more complex than generally considered. Previous studies have shown that lectins generally bind to N-linked glycans (Picton 1988), however, they do bind to some O-linked glycans as suggested by β elimination (Downs 1984). Both keratan and chondroitin sulfate polymers may have attached O- and N- linked oligosaccharides or internal binding sites (Greiling 1989, Glant 1983, Takagi 1988). Collagens have O-linked glucose and galactose side chains and may have other lectin binding sites (Nimni 1974). Other cartilage non-collagenous proteins such as chondronectin and fibronectin (Burton-Wurster 1984, 1985, Humphries 1986, Jones 1987) and glycoproteins like lubricin (Swann 1985), link protein (Glant 1982, Choi 1985, Hardingham 1982) and matrix glycoprotein (Fife 1988) also have saccharide side chains. Therefore further defining the cytoskeleton is important in understanding matrix-matrix and matrix-cell interactions. All lectin groups were expressed and it is possible to make the following conclusions:

(1) both terminal and core sequences are expressed particularly in zones I and II and the PC region,
(2) staining intensity is decreased in developing OA, reflecting increased matrix hydration,
(3) enzymatic degradations tends to lead to increased staining intensity reflecting that removal of terminal groups exposes core groups,
(4) staining in some areas is very granular as in zone II and zone V adjacent to uncalcified pegs. This suggests staining of matrix vesicles, collagen bundles seen on cross section or very large aggregated molecules,
(5) the matrix of the clones, zone V and pegs has a characteristically different staining pattern suggesting the cytoskeletal architecture is different in these areas.

5.5.3 Chondrocytes and Clones

Introduction: Both chondrocyte membrane and cytoplasm stained with a broad spectrum of lectins, suggesting the presence of structural glycoproteins. In previous studies WGA (Mallinger 1986), SBA and AHA (Schunke 1985) were negative and RCA (Yamada 1997) and UEA1 (Mallinger 1986) positive. Studies using cultured chondrocyte showed positivity of perinuclear cytoplasm with ConA, WGA and RCA (Vertel 1985). Chondrocyte from different anatomical sites may express different sugar sequence detected by some lectins but not others. LPHA differentiated costal and cephalic sternal chondrocytes, whereas GNA did not (McClure 1995).

Group 1 lectins (GNA): This lectin was not expressed suggesting mannosyl residues are not present in the cytoplasm or membrane.

Group 2 lectins (LCA, PSA, EPHA, LPHA): All lectins stained chondrocytes (matrix and peg) and clones moderately. The percentage of cells staining decreased both in depth within the matrix and with developing disease and LPHA membrane staining was absent in developed OA. These findings suggest that there is a broad expression of complex-N linked carbohydrates sequences. The absence of staining is some cells may reflect that these sequences are only expressed in metabolically active cells. EPHA stains non-bisected tri/tetra antennary sequences and therefore the production of these sequences in the cell membrane may be reduced in OA.

Group 3 lectins (STA, DSA (Group 4 also), WGA): In normals, the majority of cells showed moderate staining which was less in zones III and IV. In osteoarthrosis STA staining decreased, DSA increased and WGA was variable. Thus in normal cartilage there was good expression of both N-acetyl lactosamine and di-N-acetylchitobiose sequences, however, these residues may be lost in OA. As DSA staining was increased in OA and not STA β galactose residues may be expressed in OA. WGA staining increased after neuraminidase and aryl sulfatase. Therefore, removal of sialyl residues exposes N-acetyl lactosamine and di-N-acetyl chitobiose residues and

cleavage of sulfate groups reduces physical or ionic interference from groups to which WGA has an affinity.

Clones showed a variable pattern. DSA stained them all, STA larger clones only and WGA in severe OA only. This suggested that the ultrastructure of these chondrocytes was different to normal. The pegs stained strongly with STA and WGA but not DSA. The former two lectins have similarities in staining as they both have extended binding sites with sialic acid suggesting WGA is not exhibiting this affinity in pegs.

Group 4 lectins (MPA, ECA, CTA): With MPA and ECA chondrocytes (peg and matrix) and clones showed a similar staining pattern to Group 3 lectins with diminished staining in OA. CTA stained the cytoplasm of a small percentage of cells in zones III-IV. Therefore, the membrane and cytoplasm expressed Gal β 1,3 GalNAc α 1-, Gal β 1,4 GalNAc α 1- and GalNAc α1 sequences. The absence of matrix staining with CTA indicates subtle differences in sequences in subsets of N-acetyl lactosamine that need to be further defined. Neuraminidase digestion increased staining with all lectins thus the removal of sialyl residues reveals unexposed groups. With β elimination there was decreased staining with both ECA and MPA suggesting that there was binding to O-linked oligosaccharides. MPA with β galactose led to decreased staining confirming binding to these residues. ECA with fucosidase markedly increased cell membrane staining therefore removal of core fucosyl residues is revealing N-acetyl lactosamine sequences.

Group 5 lectins (HPA, VVA B4, WFA, DBA): Staining with this group of lectins was very heterogeneous. DBA stained cytoplasm in severe OA and VVA B4 stained both in a small percentage of cells. HPA and WFA stained cytoplasm and membrane of some cells and this increased in OA. Clones stained in some groups with WFA, in the severe group with HPA and DBA and not with VVA B4. Peg chondrocytes did not stain with DBA or HPA and to a variable degree with WFA and VVA B4. Therefore the membranes expressed N-acetyl galactosamine, internally O-glycosidic α linked GalNAc and GalNAc α 1,6 Gal β 1- sequences. Lack of membrane staining with DBA suggested that there was no α 1,2 fucosyl substitution of the sub terminal galactosyl residue in α 1,3 linkage to the terminal GalNAc in the matrix. However

matrix. However both the α 1,3/6 linkage to GalNAc are present in the matrix. Both neuraminidase and β elimination increase staining with VVA B4, DBA and WFA cytoplasm and membrane stained. Therefore, removal of sialyl and alkali labile residues reveal underlying groups. The staining pattern with these enzymatic degradations with HPA was either unaltered or slightly decreased suggesting an affinity for sugars of O-linked saccharides.

Group 6 lectins (LTA) : There was no staining in zone I, cytoplasm only in zone II and cytoplasm and matrix in zones III-IV. Clones stained in all groups and peg chondrocytes in the severe group only. There was a dramatic increase in staining with fucosidase again reflecting that removal of peripheral fucosyl sequences reveals internal core sequences. Likewise β elimination increased staining suggesting removal of alkali labile groups is revealing fucosyl containing N-linked oligosaccharides or non-cleaved O-linked oligosaccharides.

Group 7 lectins (MAA, SNA, LFA) : These lectins showed a heterogeneous spectrum of staining reflecting the different sialyl groups bound. With MAA and SNA cytoplasm and membrane stained in a few cells in the normals and in the majority of cells in the OA groups. LFA stained cytoplasm and membrane in an increasing percentage of cells across the normal, ageing, early OA group and this then decreased in the severe groups. Both peg chondrocytes and clones showed stronger staining in the OA groups. Neuraminidase and β elimination led to increased staining for the reasons previously discussed.

Summary: There was a broad pattern of staining with lectin groups 2-7 indicating that a wide number of carbohydrate sequences are expressed on chondrocytes. Variable staining of the number of cells and cytoplasm and membrane suggests some cells are metabolically active and others non-responsive. Therefore upper zone chondrocytes may be more metabolically active than those in the deep zones in normals and OA where they may become non-responsive. Both clones and peg chondrocytes showed unique staining patterns suggesting a difference in metabolic function to "normal" cells.

5.5.4 Chondro-Osseous Junction (Vessels and Splits)

Vessels and Vessel Matrix Introduction: –This section considers the staining of the plugs of vessels which are seen crossing the tidemark and extending into uncalcified cartilage (Wood 1970, Greenwald 1969, Redler 1975). These vessels are a normal anatomical feature of this region, however, increased vascularisation of this area is seen early in the osteoarthrotic process. It is possible this is a primary event or it could represent a local reparative response.

Group 1and 6 lectins (GNA, LTA): did not stain indicating that neither vessel endothelium or associated matrix expressed mannosyl or fucosyl sequences.

Group 2 lectins (LCA, PSA, EPHA, LPHA) : LCA, PSA and EPHA all stained vessels and matrix, LPHA did not. Therefore there was wide expression of complex N-linked sequences and these were all the non-bisected variants with bisected bi/tri antennary sequences not being expressed. Neither ageing or osteoarthrosis led to a great change in staining pattern.

Group 3 lectins (STA, DSA, WGA): All three lectins showed good expression and WGA showed more intense staining in developed OA. This implies the presence of N-acetyl lactosamine, di-N- acetylchitobiose and possibly sialyl sequences. The increased staining with WGA suggests that these residues may be being expressed more in OA, or alternatively, that there is a loss of sialyl residues revealing more underlying lactosamine.

Group 4 lectins (MPA, ECA, CTA): There was very little expression of these lectins. MPA stained in severe OA, ECA only after neuraminidase and CTA not at all. This indicates that in severe OA alterations in the matrix is revealing binding sites to Gal β 1,3 GalNAc α1- and GalNAc α1. Also that removal of sialyl residues is exposing underlying disaccharides with a β 1,4 linkage i.e. Gal β 1,4 GlcNAc β 1-.

Group 5 lectins (HPA, WFA, VVA B4, DBA): DBA and VVA B4 did not stain, WFA stained with all groups and the staining with HPA was variable. These features suggest the presence of GalNAc α1,6 Gal β-, GalNAC α1,3 Gal β 1- and GalNAc α1-

residues. With a lack of internal O-linked structures (VVA B4) and no terminal fucosyl residues (DBA). This latter observation was confirmed by absence of LTA staining. Enzymatic degradation did not alter staining.

Group 7 lectins (MAA, SNA, LFA): MAA and SNA were positive and LFA negative. This shows that some sialyl residues are expressed in particular NeuNAc α 2,3 Gal β 1- and its α 2,6 variant, whereas N-glycosyl neuraminic acids are not.

Splits: as with the surface of the cartilage all lectins showed staining at the horizontal tidemark splits. This is probably an edge effect and it is possible these splits are artefactual.

Summary: The vessels and vessel matrix did not stain with group 1 or 6 lectins suggesting an absence of mannosyl and fucosyl residues. Staining was otherwise suggestive of wide expression of sequences from the other groups. There was very little change in expression with ageing and osteoarthrosis, perhaps indicating "vascularisation" was a secondary phenomena.

5.5.5 Bone

Introduction: No studies that examine the lectin histochemistry of subchondral bone osteocytes or matrix were found on literature review. Chondrocytes are known to play an integral role in bone-turnover and have characteristic dendritic intracellular connections (Bonucci 1990).

Group 1, 6, 7 lectins (GNA): No staining, therefore mannosyl, fucosyl and sialyl residues are not expressed.

Group 2 lectins (PSA, LCA, EPHA, LPHA): PSA, LCA and EPHA stained both osteocytes and matrix of all groups and LPHA the severe group only. This suggests the presence of a large number of non-bisected antennary chains. In severe OA bisected chains are now expressed either due to conformational changes in the cells and matrix or due to production of new sequences.

Group 3 lectins (STA, DSA, WGA): All three lectins showed staining. That of WGA was increased with neuraminidase and in particular the dendritic processes between osteocytes were clearly demonstrated (Figures 4-100 to 4-101). Therefore there is expression of N-acetyl lactosamine, di-N-acetylchitobiose and possibly sialyl sequences. As staining is increased with neuraminidase this negates against sialyl expression as their removal appears to be revealing additional concealed N-acetyl lactosamine sequences.

Group 4 lectins (MPA, ECA, CTA): Both MPA and CTA stained strongly whereas ECA was negative. MPA clearly demonstrated the dendritic processes between osteocytes. This pattern was not substantially altered by enzymatic degradations. These patterns suggest β galactose sequences are being detected on Gal β 1, 3/4 GalNAC β 1- and GalNAc α 1. Lack of staining with ECA suggests that there may be substitution of β galactosyl residue.

Group 5 lectins (HPA, WFA, VVA B4, DBA): DBA, VVA SB4, HPA did not stain and WFA stained with increasing intensity across the group. With HPA and neuraminidase there was a dramatic increase in osteocytes staining and dendritic processes were prominent. This suggests there was expression of GalNAc α 1,6 Gal β 1- and/or the α 1,3 variant. HPA is not staining GalNAc α1- until sialyl residues were cleaved suggesting that the residue was being concealed.

Summary: Both bone matrix and osteocytes showed a wide range of staining with Group 1-5 lectins. This reflects the presence of a wide variety of carbohydrate residues on proteoglycan aggregates. Other glycoproteins found in bone matrix also bear oligosaccharides including osteonectin, osteopontin and bone sialoprotein (Engel 1987, Hughes 1987). Of particular note was the presence of very prominent osteocyte dendritic process which stained clearly with WGA, MPA and HPA. In particular, with HPA these dendritic processes were seen abutting onto both uncalcified cartilage pegs and bone marrow spaces. This adds further weight to the evidence that the microanatomy of this region is much more complex than has been previously considered.

5.6 Issues

This research has undertaken to cover a broad spectrum of topics in relation to the normal anatomy of articular cartilage and its lectin histochemistry. In retrospect, it is possible to make a number of comments which would have further strengthened the observations.

Collecting human tissue for research is increasingly difficult in light of the general awareness of medico-legal issues. The material for this research was collected from hospital postmortems and under the UK Anatomy Act it was possible to gather material for research. Unfortunately the number of specimens collected for the normal and ageing groups was relatively small and larger numbers would have added strength to the arguments put forward and allowed for more extensive studies. Future research would benefit from collecting cases from the Coronial system through which it would be possible to obtain more samples in the normal group for example from motor vehicle accidents, industrial accidents and suicides. In both the United Kingdom and Australia the coroners have a positive attitude towards ongoing medical research, however, the current Coroner's Acts allow for material to be collected only for the purposes of providing a finding to the coroner. Approached in an appropriate and understanding manner, relatives in some cases would allow materials to be harvested for research and this is an issue worthy of pursuit.

All examination of tissue and scoring were undertaken by the author. There would be benefits with scoring by a second party to ensure consistency of scoring parameters. As a broader part of the study uncalcified cartilage was collected which was used by others in micromechanical studies (Roberts, 1996). In particular, repeating the S100 study on uncalcified tissue would be interesting given S100 proteins putactive role in calcification, however, this would have the problem that the tidemark area could not be assessed.

5.7 Future Directions

This thesis has been extensive, but not exhaustive in its review of cartilage, microanatomy and lectin histochemistry and a number of new avenues of research have been opened.

The study has demonstrated that there appear to be clear macroscopic and microscopic differences between ageing and early osteoarthrotic cartilage using microscopic parameters. As the scoring system was semi-quantitative and statistical standards have not been applied. As these findings have implications in relation to understanding of these pathological processes and possible treatment regimes, further defining these differences would be a worthwhile research project. A histomorphometric study of the matrix, chondrocyte, clone and chondro-osseous changes in normal, ageing and early osteoarthrotic cartilage with larger cohorts would permit more formal statistical analysis. The application of multivariate analysis techniques would more clearly define the parameters which characterise these processes.

Although the three dimensional reconstruction studies undertaken clearly demonstrate the presence of uncalcified cartilage, pegs connecting with the underlying bone marrow spaces these studies were relatively limited in their extent. The field of three dimensional reconstruction of human tissues is currently burgeoning given the meteoric advances in computer technology. A study undertaken with a greater number of serial sections would better be able to demonstrate the three dimensional architecture of this region. More advanced mathematical algorithms which have been developed for the computerised tomography field which allow 360 degree visualisation of reconstructed images increasing an understanding of the anatomy.

The studies on lectin histochemistry have shown that there are characteristic differences in staining patterns in anatomical regions of cartilage and that these are altered in both ageing and osteoarthrosis. Previous studies have shown that in addition to O- and N-linked oligosaccharides lectins may have an affinity for carbohydrates on collagen, chondronectin, fibronectin and other matrix components. Although it is possible to predict the carbohydrate sequences which are being stained by lectin histochemistry it is not always possible to conclude which macromolecules are being bound. Further information would be obtained by utilising a broader panel of lectins so that known sequences on glycoproteins, proteins etc, may be defined and individual molecules identified. The utilisation of proteolytic enzymes to cleave off molecules and of lectin affinity chromatographic techniques and subsequent

biochemical analysis would allow molecules to be identified. The use of immunohistochemical and insitu hybridisation studies would allow postulated lectin binding sites to be confirmed. Both the tidemark and osteocytes showed very specific characteristic binding patterns and electron microscopic and scanning electron microscopic studies of these area with gold bound lectins would further our understanding of the submicroscopic structure of these areas.

A broader panel of lectins than any previous study in the osteoarticular field was used and has demonstrated a number of unique observations. This panel of lectins would be extremely useful in animal models of osteoarthrosis to assess the validity of therapeutic agents in proven models of osteoarthrosis. Therefore further studies using this panel of lectins and defining staining patterns in experimental animal models of osteoarthrosis would provide a useful experimental baseline for assessing developing OA and therapeutic regimes. In addition the same model might prove useful in the role of cultured chondrocytes and osteochondral grafts in cartilage defects. The field of osteoarticular pathology continues to expand and this research has extended the avenues for future exploration.

5.8 Conclusions

The following main conclusions result from the current research:

1. That ageing and early osteoarthrotic cartilage show characteristic differences in their macroscopic and microscopic features. Although alterations in matrix staining intensity and chondrocyte architecture occur in both they are much more pronounced in early osteoarthrosis. In the latter there is very marked loss of glycosominaglycans in the superficial deep zones and a prominent alteration in chondrocyte architecture with early clone formation, acellular areas and disruption of the zone IV chondrocyte columns. These ultrastructural changes lead to macroscopically visible alterations in mechanical properties with a poorer viscoelastic response in early osteoarthrosis.
2. The alterations seen in matrix and chondrocytes continue as the disease process evolves into moderate and severe osteoarthrosis. There are very marked changes in glycosominoglycan staining and a profound alteration in chondrocytes with the formation of massive multinucleated clones.

3. Characteristic alterations are seen in the pattern of S100 protein staining in early osteoarthrosis. There is an increased staining intensity in chondrocytes and pericellular matrix and where present clones show positivity for this protein. The physiological function of S100 protein in chondrocytes has not been clearly defined.
4. It is postulated that the anatomy of the chondro-osseous region is more complex than previously documented. These studies clearly demonstrate the presence of pegs of uncalcified cartilage faithfully followed by the tidemark dipping through calcified cartilage and abutting onto marrow spaces. Lectinhistochemical studies exhibit a unique combination of carbohydrate sequences within the tidemark and the presence of osteocyte dendritic processes extending to these uncalcified pegs and marrow spaces. These findings must alter our view of the current concept of cartilage nutrition. They indicate that cartilage my receive nutrition directly from bone marrow spaces and that a direct physiological connection exists between osteocytes and uncalcified articular cartilage.
5. Lectin histochemistry of articular cartilage demonstrates that our current understanding of cartilage carbohydrate chemistry is still insufficient. The main findings of the thesis in regard to lectins are:

- Some lectins do not stain at all, therefore certain saccharide sequences are not expressed.
- Matrix staining is varied in normals, in zones and regions and that there are characteristic alterations in ageing and osteoarthrosis.
- The staining of cell membranes and cytoplasms is varied both in anatomical regions and disease processes suggesting that some cells may be metabolically active and other in a non-responsive phase. Cells may have different phenotypic expressions and these may differ throughout cartilage and alter in ageing and OA.
- The tidemark shows a unique pattern of lectin histochemistry suggesting that it is biochemically quite distinct from the adjacent uncalcified and calcified cartilage zones.
- The Zone V matrix shows a different pattern of staining from the uncalcified matrix suggesting alterations in the glycan cytoskeleton in relation to calcification. Also the uncalcified cartilage in pegs stains differently from the adjacent zones I - IV indicating that there may be marked differences in matrix ultrastructure,

possibly associated with calcification mechanisms or a role in the nutrition of the overlying cartilage.

- Some lectins stain osteocytes and their interconnecting cytoplasmic dendritic processes which in places are abutting on to uncalcified cartilage pegs and marrow spaces therefore that they may be intimately involved in normal cartilage physiology.
- These findings reaffirm our current understanding that the carbohydrates of glycoproteins and glycosaminoglycan polymers play a significant role in the microenvironment of articular cartilage and subchondral bone.

Articular cartilage provides a unique role in maintaining mobility as demonstrated by the incapacity and morbidity that result from osteoarthrosis. The chondro-osseous region is more complex than previously described. A greater understanding of this region and of cartilage carbohydrate chemistry may enhance our understanding of osteoarthrosis and assist in developing treatment strategies for this common articular disease.

6 CHAPTER 6: REFERENCES

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7 CHAPTER 7: APPENDICES

7.1 APPENDIX A Haematoxylin and Eosin (H&E) Stain

	Solution	Time
1.	Xylene	2 mins
2.	Xylene	2 mins
3.	100% alcohol	2 mins
4.	100% alcohol	2 mins
5.	95% alcohol	2 mins
6.	70% alcohol	2 mins
7.	Wash in running water until clear	
8.	Stain in Mayers Haematoxylin	5 mins
9.	Wash in running tap water	5 mins
10.	Stain in 1% Eosin	2 mins
11.	Wash in running tap water	30 secs
12.	70% alcohol	30 secs
13.	95% alcohol	1 min
14.	100% alcohol	2 mins
15.	100% alcohol	2 mins
16.	Xylene	2 mins
17.	Xylene	2 mins
18.	Mount under coverslip using dpx mount	

7.2 APPENDIX B Toludine Blue Stain

	Solution	Time
1.	Xylene	2 mins
2.	Xylene	2 mins
3.	100% alcohol	2 mins
4.	100% alcohol	2 mins
5.	95% alcohol	2 mins
6.	70% alcohol	2 mins
7.	Wash in running water until clear	
8.	Stain in toludine blue	30 secs
9.	Wash in running water until clear	
10.	Drain & blot dry with filter paper	
11.	Air dry for a minute or two	
12.	Xylene	2 mins
13.	Xylene	2 mins
14.	Mount under coverslip using dpx mount	

7.3 APPENDIX C Safranin 'O' Stain

	Solution	Time
1.	Xylene	2 mins
2.	Xylene	2 mins
3.	100% alcohol	2 mins
4.	100% alcohol	2 mins
5.	95% alcohol	2 mins
6.	70% alcohol	2 mins
7.	Wash in running water until clear	
8.	Safranin 'O'	5 mins
9.	Wash in running water until clear	
10.	Fast Green	20 secs
11.	Wash in running water until clear	
12.	70% alcohol	30 secs
13.	95% alcohol	1 min
14.	100% alcohol	2 mins
15.	100% alcohol	2 mins
16.	Xylene	2 mins
17.	Xylene	2 mins
18.	Mount under coverslip using dpx mount	

7.4 APPENDIX D Alcian blue stain

Preparation of salt solutions

Final $MgCl_2$ Molarity	0.05	0.5	0.9
1M acetate buffer pH5.8	2.5ml	2.5ml	1.5ml
5M $MgCl_2$	1.0ml	10ml	18ml
Water to	50ml	50ml	50ml

Method

1. Make up solutions as shown above.
2. Take sections to water.
3. Mix 25ml salt solutions with 25ml 0.5% (w/v) alcian blue 8 GX in water.
4. Stain sections overnight in dye/salt solutions.
5. Rinse sections in buffered salt solution, 25ml salt solution and 25ml water for 5 mins.
6. Rinse for 5 mins in water.
7. Dehydrate clear and mount.

7.5 APPENDIX E Picro sirius red stain

Preparation of celestine blue stain

Dissolve 50g ferric ammonium sulphate in 1 litre cold water.

Add 5g celestine blue B and boil for 3 mins.

Filter when cool and add 140ml glycerol.

Preparation of Mayer's haematoxylin

Dissolve 1g haematoxylin, 50g aluminium potassium sulphate and 0.2g sodium iodate in 1 litre water.

Add 50g chloral hydrate and 1g citric acid.

Boil mixture for 5 mins.

Cool and filter.

Preparation of Picro sirius red

0.1% (w/v) sirius red dissolved in saturated aqueous picric acid.

Method

1. Take sections to water.
2. Stain nuclei in celestine blue for 5 mins.
3. Blue in water.
4. Stain in Mayer's haematoxylin for 5 mins.
5. Wash in running tap water for 30 mins.
6. Stain in picro sirius red for 30 mins.
7. Dehydrate rapidly in three changes of absolute alcohol.
8. Clear and mount.

7.6 APPENDIX F

S100 Staining

1. Place in Tris buffered saline (TBS) for 5 minutes.
2. Incubate for 20 minutes with normal swine serum diluted 1:5 in TBS for blocking of non-specific background.
3. Tap off serum and wipe away the excess.
4. Incubate for 20 – 30 minutes with the rabbit polyclonal antibody diluted optimally in TBS.
5. Tap off antibody and place slide in TBS bath for 5 minutes.
6. Incubate for 20 – 30 minutes with swine anti-rabbit immunoglobulins/biotinylated diluted 1:300 – 1:800 in TBS.
7. Tap off biotinylated antibody and place slide in TBS bath for 5 minutes.
8. Incubate for 20 – 30 minutes with avidin/AP diluted 1:100 – 1:300 in TBS.
9. Tap off conjugate and place slide in TBS bath for 5 minutes.
10. Incubate for 10 – 20 minutes with an alkaline phosphatase substrate solution.
11. Rinse with distilled water.
12. Counterstain and mount with coverslip.

7.7 APPENDIX G

Lectin staining

1. Cut sections and dry at 70°C in oven.
2. Dewax and take to 99% alcohol.
3. Block endogenous peroxidase activity for 30 mins in 400ml methanol with 0.4% (v/v) IM hydrochloric acid and 2ml 30% (v/v) hydrogen peroxide.
4. Rinse briefly in tap water then in three changes of TBS (0.05M TRIS Buffered Saline, pH 7.6) for 2 mins each.
5. Warm in TBS at 37°C for 3 mins then trypsinise at 37°C for 10 mins in 300ml TBS containing 300mg calcium chloride & 100mg crude trypsin.
6. Wash briefly in cold running water to stop the reaction.
7. Wash in three changes of TBS for 5 mins each.
8. Remove excess TBS, place slides in a wet box and pipette on the biotinylated lectins (10μg/ml), (with a negative control containing only diluent – TBS with 1mM calcium chloride). Leave for 30 mins.
9. Gently rinse off lectins with TBS + $CaCl_2$, and wash in three changes of TBS + $CaCl_2$ for 5 mins. each.
10. Remove excess TBS, replace in the wet box and pipette on avidin peroxidase, at 5 μg/ml in **0.125 M** TBS, pH 7.6, containing 0.374M sodium chloride. Leave for 60 mins.
11. Gently rinse with TBS (0.05M) and wash in three changes of TBS for 5 mins. each.
12. Develop in DAB solution (150mg 3,3-Diaminobenzidine tetrahydrachloride dihydrate in 300ml TBS with 45μl of 30% hydrogen peroxide added just before use).
13. Wash briefly in tap water.
14. Counterstain in 0.25% (w/v) methyl green for 30 secs.
15. Wash, dehydrate, clear and mount.

7.8 APPENDIX H

B – galactosidase pretreatment of paraffin sections

B – galactosidase Grade VIII from E.Coli **G 5635** (Sigma Chemical Co)

O – Nitrophenyl – B – D galactopryanoside (Koch – Light Laboratories, Coinbrook, U.K.)

N.B. These substances may be harmful by ingestion, inhalation and skin contact. Please take relevant safety precautions, i.e. wear latex gloves, wipe up spillages, etc.

1. Dewax sections.
2. Block endogenous peroxidase.
3. Trypsinise if necessary.
4. After washing in T.B.S. rinse briefly in 0.1M Phosphate buffer pH 7.3

Phosphate buffer

A – 0.2M Sodium Dihydrogen orthophosphate (irritant!)

M.W. 156

3.12g in 100ml distilled water

B – 0.2M diSodium Hydrogen orthophosphate (irritant!)

M.W. 142

2.83g in 100ml distilled water

mix 11.75 ml A + 38.25 ml B make up to 100 ml and pH to 7.3

5. Incubate sections in 10 units/ml B – galactosidase in 0.1M phosphate buffer pH 7.3, with 1.1×10^{-6} moles / ml magnesium chloride and a trace of 2 – mercaptoethanol at 37°C.

7.9 APPENDIX I

α - L - Fucosidase pretreatment of paraffin sections

α - L – Fucosidase from bovine kidney F 5884 (Sigma Chemical Co)

pNitrophenyl α - L – Fucopyranoside N 3628 (Sigma Chemical Co)

N.B. These substances may be harmful by ingestion, inhalation and skin contact. Please take relevant safety precautions, i.e. wear latex gloves, wipe up spillages, etc.

1. Dewax sections.
2. Block endogenous peroxidase.
3. Trypsinise if necessary.
4. After washing in T.B.S. rinse briefly in Phosphate buffer pH 6.5

Phosphate buffer

A – 0.2M Sodium Dihydrogen orthophosphate (irritant!)

M.W. 156

3.12g in 100ml distilled water

B – 0.2M diSodium Hydrogen orthophosphate (irritant!)

M.W. 142

2.83g in 100ml distilled water

mix 33.95 ml A + 16.05 ml B make up to 100 ml and pH to 6.5

5. Dilute Fucosidase to 0.1 unit/ml in phosphate buffer pH 6.5
6. Pipette Fucosidase onto sections and incubate at 24°C for 15 mins. After 15 mins. collect excess enzyme with a Pasteur pipette and place in a tube (**tube** 1).
7. Without washing, repeat step 6 for a total of 4 times, i.e. 1 hour incubation.
8. Wash sections in distilled water and T.B.S. (3 x 2 mins).
9. To the enzyme collected after each incubation (**tubes 1,2,3 and 4**), add p-Nitrophenyl - α - L – Fucopyranoside, made up in phosphate buffer pH 6.5 (The concentration of this solution is **not** important, i.e. add a small amount of powder to a test tube of buffer).

Mix approx. equal parts of collected enzyme in tubes 1 – 4 and p-nitro solution in phosphate buffer. Leave for 15 mins at 24°C. The mixture should turn yellow, if it has not, add a small amount of concentrated sodium hydroxide (corrosive and toxic!)

10. Proceed with lectin method as normal, i.e. biotinylated lectin, avidin peroxidase and D.A.B.

7.10 APPENDIX J

Aryl– Sulfatase pretreatment of paraffin sections

Use aryl – sulfatase sulfohyrolase from limpets (patella vulgata) (sigma cat no. S – 8629)

Make up to 125 unit/ml with distilled water and store at -20°C.

When ready for use dilute to 2.5 units/ml in acetate buffer pH 5.0

(aryl sulfatase is an irritant!)

0.2 M acetate buffer pH 5.0:

Stock A – 0.2 M acetic acid (corrosive and flammable!)

Stock B – 0.2 M sodium acetate

To make up use 14.8ml of A + 35.2ml of B and make up to 100ml with distilled water.

1. Dewax sections and take to 99% methylated spirits.
2. Block endogenous peroxidase with hydrogen peroxide.
3. Wash (3 x TBS)
4. Trypsinise if required (wash 3 x TBS)
5. Wash in distilled water.
6. Wipe of excess water.
7. Pipette on 2.5 units/ml aryl sulfatase made up in acetate buffer pH 5.0.
8. Incubate for 1 hour at 37°C.
9. Rinse in distilled water wash in TBS and continue staining.

7.11 APPENDIX K

β Elimination (Downs 1973)

(Paraffin sections)

N.B. Sections tend to detach with this method. Be sure to use an adhesive and treat very carefully, especially with the first HCL wash.

1. Dewax, block endogenous peroxidase and trypsinise if necessary.
2. Rinse in TBS – 3x2 mins.
3. Rinse briefly in distilled water.
4. Make up the following mixture and leave in the water bath at 45°C to warm up:-

150ml	or	50ml	DMSO (Dimethylsulphoxide)
120ml		40ml	distilled water
30ml		10ml	99% methylated spirit
2.86g		0.954g	Potassium Hydroxide (final concentration is 0.17M)
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300ml		100ml	
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Extreme care must be taken with DSMO as it is a carcinogen, may cause adverse mutagenic or teratogenic effects. It freely penetrates skin and eyes and is harmful by ingestion. Symptoms include nausea, vomiting, cramps and lethargy. Has caused corneal opacity in experimental animals. Allergenic. Always wear gloves and use in fume cupboard. DISPOSE OF DMSO IN SMALL QUANTITIES DOWN THE SINK WITH PLENTY OF WATER. 99% methylated spirit (74o.p. industrial methylated spirit) is harmful, irritant and flammable! Potassium Hydroxide is corrosive and toxic!

5. Incubate sections for 40 – 60 mins in the above solution at 45°C.
6. Very carefully wash to neutralise in 10mM HCL (1ml N HCL in 100ml distilled water) x 2.
7. Rinse in 0.1M sodium phosphate buffer pH 7 (or PBS).
8. Rinse in distilled water, followed by TBS before staining with lectin as normal.

7.12 APPENDIX L

Neuraminidase Digestion of Paraffin Sections

Use neuraminidase from clostridium perfringens, type VI (Sigma Cat No. N 3001.), obtained as a lyophylised powder. Make up to 1 unit / ml with distilled water and store at -20°C.

When ready for use, dilute to 0.1 unit/ml in neuraminidase buffer
(neuraminidase is harmful and an irritant!)

Neruaminidase buffer: 0.2 acetate buffer pH 5.5 containing 1% calcium chloride.
Stock A – 0.2 M acetic acid (corrosive and flammable!)
Stock B – 0.2 M sodium acetate.

To make up use – 11 ml of A + 89 ml of B.
Adjust pH to exactly 5.5 with the above solutions, then add 1% calcium chloride and store in fridge.

1. Dewax sections and take to 99% methylated spirits.
2. Block endogenous peroxidase with hydrogen peroxide.
3. Wash (3 x TBS)
4. Trypsinise if required (wash 3 x TBS)
5. Wash in distilled water.
6. Wipe of excess water.
7. Pipette on 0.1 unit/ml neuraminidase made in 0.2 M acetate buffer pH 5.5 containing 1% calcium chloride.
8. Incubate for 1 hour at 37°C.
9. Rinse in distilled water wash in TBS and continue staining.

7.13 APPENDIX M

Materials and Suppliers

1. Agar Scientific Ltd., Stanstead, Essex – Glutaraldehyde (25% (w/v) practical grade).
2. Aldrich Chemical Co. Ltd. Dorset – Diaminobenzidine tetrahydrochloride, 1,9-dimethylmethylene blue.
3. Amersham International, Aylesbury, Buckinghamshire D[U^{14}C]-galactose, sodium [^{35}S] – sulphate.
4. Bio-Rad Laboratories Inc. Hemel Hempstead, Herfordshire-Bio-Gel P4, sodium cacodylate.
5. Boeringer Mannheim Ltd., Lewes, East Sussex-Boitinylated lectins – DSA, GNA, MAA, SNA.
6. BDH, Merck Ltd., Lutterworth, Leics (analar grade except where indicated) Acetic acid, acetone, alcian blue 8GX, aluminium potassium sulphate, ammonium ferric sulphate, calcium chloride, celestine blue B, citric acid, ethanol, formaldehyde, formic acid, glycerol, haematoxylin, hydrochloric acid, magnesium chloride, pararosaniline, periodic acid, picric acid, safranin O, sirius red, sodium acetate, sodium azide, sodium formate, sodium hydroxide (pellets), sodium iodate, sodium metabisulphite, sodium sulphate, sulphuric acid, TRIS.
7. Falcon, Becton Dickenson, Cowley, Oxford – 35mm sterile culture dishes (cat. No. 1008 – non-tissue culture treated).
8. Fluka Chemicals/Biochemicals, Glossop, Derbyshire – Sodium nitrite.
9. Genta Medical, York – Industrial methylated spirit (99%, 74°P), xylene.
10. Gibco BRL, Life Technologies Ltd., Paisley – 10xDMEM, Foetal calf serum, 100-L-glutamine, 10x PBS, 100-penicillin/streptomycin, 7.5% sodium bicarbonate, 10-trypsin.
11. ICN Biomedicals, Irvine, Ayrshire, Flowpore 0.22μm filters.
12. R.A.Lamb, London – DPX (mountant)
13. May & Baker Ltd, Dagenham – Lithium carbonate
14. Medicell International Ltd., London – Dialysis tubing
15. National Diagnostics, New Jersey, U.S.A. Ecoscinct, A, Uniscinct BD.
16. Pharmacia Biotech Ltd., Milton Keynes- Blue-Dextran 2000, Sephadex G-75, Sepharose CL-2B.

17. Sarstedt, Germany – Disposable cuvettes (cat no. 67.742).
18. Sigma Chemical Co. Ltd. Poole, Dorset – E Amino-n-caproic acid, L-ascorbic acid, avidin-peroxidase, biotinylated lectins – (AHA, BSA-1B4, CTA, DBA, ECA, HPA, LCA, LEA, LTA, MPA, ePHA, lPHA, PSA, SBA, UEA-1, WFA), charcoal, chloral hydrate, chondroitinase ABC, chondroitin sulphate C, collagenase type 1A, ethylenediamine-tetraacetic acid, N-ethylmaleimide, fluorescein diacetate, guanidine hydrochloride (99+% & practical grade), hyaluronic acid, hyaluronidase, phenylmethylsulphonyl fluoride, sodium iodoacetate, toluidine blue, trypsin-type II crude (in lectin binding).
19. Vector Laboratories, Peterborough – Biotinylated lectin STA.

7.14 APPENDIX: RECENT ADVANCES IN HUMAN OSTEOARTHROSIS

Osteoarthrosis continues to remain a disease process with a significant financial burden on societies around the world and causes major morbidity in particular when larger joints such as the knee and hip are affected. In the last 5 years there have been important advances in noninterventional and diagnostic techniques, new and innovative treatment regimes and in our understanding of the biochemical events occurring in the cartilage matrix and chondrocytes. This Appendix summarises these recent advances.

Pathogenesis: In the last few years there has been a heightened awareness to the concept of early osteoarthrosis and its development in those who partake in regular competitive sport (Buckwalter 1997, Roos 1998). Such activities subject joints to repetitive high levels of impact and torsional loading increasing the risk of articular cartilage degeneration particular through early disruption of the collagen (Prockop 1998). There has also been an increased understanding of managing osteoarthrosis in the elderly with an appreciation that there is a need to maintain a moderate level of fitness combined with early interventional treatment (Yasuda 1997, Ling 1998). The use of 3-dimensional computational models of the human knee joint (Li 1999) have helped to further understand the degenerative processes that leads to a breakdown of the normal load-bearing capacity of articular cartilage (Cohen 1998). The resulting changes in the viscoelastic properties result from an alteration in the extracellular matrix (Mow 1999) with subsequent swelling and changes in the biochemical properties of the cartilage (Chen 1999). Undoubtedly deformation of chondrocytes lead to changes in their metabolic activity (Guilak 1999). Subsequent alterations in chondrocyte–matrix interactions (Buckwalter 1998) may then lead to abnormal production of cytokines, nitric oxide and other inflammatory mediators which alter cartilage homeostases (Lotz 1999, Martel–Pelletier 1999 A and B). These inflammatory mediators may have a significant effect on extracellular nonstructural matrical proteins, particularly those involved in proteoglyan aggregation (Watanabe 1998). The matrix metalloproteinases have always been considered to have a key role in precipitating cartilage degradation (Skotincki 1999). However, there is a heightened awareness of the importance of aggrecanases and their role in the breakdown of the major aggregating proteoglycan protein aggrecan (Little 1999). Aggrecan is one of the major structural components of cartilage, its globular and

glycosaminoglycan rich domains binding hyaluronam and link proteins to form huge aggregates (Watanabe 1998). Aggrecanases are a group of enzymes involved in cartilage matrix turnover and breakdown. They cleave the interglobular domain of the aggrecan core protein leading to increased matrix hydration and altered viscoelastic properties (Little 1999).

A greater understanding of aggrecan and other recently discovered noncollagenous nonproteoglycan macromolecules including chondromodulin, pleiotrophin (Neame 1999) and the matrilins (Deak1990) will further our understanding of matrix-matrix and matrix-cell interactions in normal and osteoarthrotic cartilage.

Recent research also indicates that subchondral mineralised tissues play a pivotal role in the development of osteoarthrosis (Burr 1997). It is suggested that altered joint biomechanics may lead to a change in cartilage-bone interrelationships (Matsui 1997) with increased osteoblastic and osteoclastic activity triggered by the release of local growth factors resulting in changes in bone density (Dequeker 1997). Continued developments in these fields will provide further understanding of osteoarthrosis hopefully opening avenues for new therapeutic regimes.

Diagnosis and treatment: The last five years have seen advances made in the use of modern diagnostic imaging tools when applied to assessment of osteoarthrotic cartilage. Although plain radiography remains the cornerstone of diagnosis (Mazzuca 1999) there is an increased awareness that high-resolution magnetic resonance imaging provides more accurate resolution and good correlation with histological and pathological parameters (Goodwin 1998, Frank 1999, Waldschmidt 1999) Both high-frequency ultrasound (Toyras 1999) and high-resolution ultrasound (Lefebvre 1998) provide useful data for 3-dimensional reconstruction of articular cartilage lesions. Increased use is being made of biological markers of osteoarthrosis improving the ability for therapeutic monitoring and follow-up of patients. In particular there is a high level of focus on hyaluronates and cartilage oligomeric matrix protein (products of enzyme catabolism of the matrix) in monitoring the extent of matrix degeneration (Woitge 1999). Treatment regimes have centred on conservative management including the benefits of weight loss (de-Leiva 1998), replacement of matrix components such as glucosaminosulfate (Gottlieb 1999) and of stimulation of articular cartilage chondrocytes by growth factors such as Human Bone

Morphogenetic Protein-2 (HBMP-2) (Sellers 1997). Allograft chondrocytes stimulated by HBMP-2 show increased metabolis activity and greater release of matrix components improving matrix integrity in the repair of osteochondral defects in osteoarthritic joints. Advances continue to be made in interventional procedures, particularly in our understanding of the immunology of chondrocyte transplantation (Jackson 1996) and in the use of autologous articular chondrocytes (Wakitani 1998) and chondrocyte-seeded collagen matrices (Nehrer 1998) both used in the repair of chondral defects.

With our understanding of the human genome approaching the potential for the use of gene therapy in the management of osteoarthrosis becomes an increasing reality in the relatively near future (Evans 1999). Genes whose products stimulate chondrogenesis or inhibit breakdown of the cartilagenous matrix are candidates for therapeutic use. These genes may be transferred to the synovium or cartilage of affected joints by in vivo or ex vivo means using a variety of vectors. Transfer of such genes to chondroprogenitor cells is also a particularly attractive approach.

The next three sections comment on recent advances in specific areas of this thesis in particular tidemark, lectin histochemistry and S100 protein.

Tidemark: The functional importance of the tidemark has been further reaffirmed by its demonstration early in the foetal development of rabbit knee joints (Bland 1996) an observation confirmed by underdevelopment in unweighted knees of adult rats (O'Connor 1997). The effects of altered mechanics to the pathophysiology of the tidemark is further demonstrated with tidemark reduplication seen in an experimental model of osteoarthrosis including; osteoarthrosis in adult rabbits induced by a longitudinally applied force across the knee joint – distraction model (Hung 1997) and in dysbaric osteonecrosis observed in adult sheep exposed to compressed air (Lehner 1997). Alterations in sub-chondral bone density initiated by local growth factors may cause tidemark reduplication (Dequeker 1997) through changes in the biomechanics of the subchondral zone. Further such growth factors may trigger reactivation of the chondrocytes of the calcified cartilage with subsequent tidemark disruption (Oegema 1997) and also induce the neovacularization which was documented in the section on early osteoarthrosis in this thesis. Recent work on

equine arthrosis confirmed such disruption in the tidemark of adult horses with early osteoarthrosis (Norrdin 1999). An increased understanding of the growth factors and cytokines that lead to this process of neovascularization has been obtain through *in vitro* experiments using cultured endothelial cells (Diaz-flores 1994). Horizontal splitting of the tidemark was reported in this thesis early in the osteoarthrotic process and studies in Golden Syrian hamsters show defects occurring as part of early aging changes (Otterness 1999).

A functional weakness of this zone was confirmed by in vitro experiments in shear deformation of osteochondral junctions illustrated by fracturing of the tidemark zone (Broom 1996). Work on the histiological preparation of osteochondral material suggests that these osteochondral defects are best demonstrated with the use of a paraffin method with methyl alcohol as the clearing agent (Atkinson 1999). Only one paper has documented the use of 3-dimensional reconstruction techniques in understanding tidemark pathophysiology. This paper demonstrates differences in irregularity in weight-bearing and less weight-bearing regions of normal human femoral heads (Teshima 1999). No further studies appear to have been undertaken to clarify the role of the chondroclastic multinucleate giant cells described at the tidemark in advance of the vascular arcades. Similar multinucleated cells are described as being effected by growth factors at the osteochondral junction during skeletal development (Soderstron 1999) suggesting that perhaps precusor cells are reactivated by cell mediators released early in osteoarthrosis.

Lectins: No further investigations have been identified in relation to lectin histochemistry and human knee joint osteoarthrosis. A number of animal studies demonstrated differences in lectin binding of different cartilages; hamster epiglottic and cricoid cartilage (Melgrejo 1999) , bovine hyaline cartilage (Della-rocca 1995), human cricoarytenoid cartilage (Paulsen 1999), rat perichondrium (Zschabitz 1995) and in cultured sternal chondrocytes (McClure 1997). However, no significant advances appear to have been made in the fundamental understanding of the role of endogenous lectins in cartilage carbohydrate biochemistry. The lectins used in this study were predominantly exogenous plant lectins; endogenous human lectins clearly have indispensable biological functions in maintaining matrix integrity. Cartilage-derived, C-type lectin related to the serum protein, tetranectin appears to have a role in maintaining matrix integrity (Neame 1999). The aggregating proteoglycans

(aggrecan, versican, neurocan and brevican) are important components of extracellular matrices. Their N-terminal globular domain binds to hyaluronan, while their C-terminal region contains a C-type lectin domain which is involved in recognition processes decoding biological information (Zschabitz 1998, Aspberg 1999). Intracellular endogenous lectins are thought to be associated with N-linked glycoprotein traffic and the expression of complex glycoconjugates (Yamashita 1999, Zschabitz 1999).

Other observations suggest that certain exogenous lectins may have homology with endogenous lectins. For example concanavalin A has been demonstrated to cause chondrocyte hypertrophy and matrix calcification in chondrocyte culture studies (Yan 1997). Lectins clearly remain an exciting research tool for further enhancing our understanding of matrical carbohydrate biochemistry.

S100 Protein: No further additional studies appear to have been reported utilising S100 protein. Two studies were identified in animal models, both demonstrating the presence of this protein in normal human, pig, rat and mouse articular cartilage (Rucklidge 1996, Eerola 1998) .

This appendix highlights advances that have been made in the osteoarticular field in the last five years. Studies in the field of the tidemark and lectins reconfirms observations reported in this thesis.

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